

Private embryos, public policy

In the United States, assisted reproduction and embryo research in the private sector have been left largely unregulated, whereas federally funded labs face stringent controls. The distinction makes little sense.

As *Nature* went to press, US voters were going to the polls for the mid-term elections. Embryo research, stem cells and cloning have not been hot campaign topics. But when the victors take their seats in Congress, researchers can be sure that these issues won't stay off the agenda for long.

The Senate has so far failed to pass legislation on cloning to produce either babies or embryonic stem cells. But Senator Sam Brownback (Republican, Kansas), who opposes 'therapeutic cloning', as the latter practice is dubbed, is still committed to the issue. And if, as is feared, the renegade Italian scientist Severino Antinori soon claims that he has cloned a human, advocates of therapeutic cloning face a rough ride. Even if evidence for his claim is not forthcoming, it will not stop Brownback and his allies from seizing an opportunity to pass legislation to restrict a potentially important area of biomedical research.

Scientists have worked with advocates for patients to convince senators such as Dianne Feinstein (Democrat, California) and Orrin Hatch (Republican, Utah) to fight attempts to outlaw therapeutic cloning. Now, however, they should return to the political fray with an expanded vision: to help draft legislation that regulates the broad sweep of embryo research, as well as some aspects of assisted reproduction, in both public and private sectors.

Currently, the private sector is left largely to its own devices. *In vitro* fertilization clinics, in particular, have been considered off-limits. The right of an individual to reproduce, while not actually enshrined in the US constitution, has generally been held to be inalienable.

But the sands may be shifting. The Bush administration's move to extend the remit of an advisory panel charged with protecting the interest of human subjects in clinical research to cover embryos and fetuses (see page 3) suggests that it is looking again at its hands-off attitude to the private sector — the panel can recommend changes to Food and Drug Administration policies, which affect private-sector researchers. And the President's Council on Bioethics is considering whether changes are needed to the regulatory framework for embryo research.

Individual states have also begun weighing in on the matter. In September, the California legislature passed a law affirming that researchers there can derive new embryonic stem-cell lines, including from cloned embryos, as long as they do not use federal money. Other states are talking about passing similar laws. And while this may seem to benefit researchers, states emboldened by passing such legislation might in future enact restrictive laws on other aspects of research.

At the federal level, meanwhile, the freedom to conduct embryo research lies at the whim of the current and future administrations. Federally funded scientists can now work with certain prescribed embryonic stem-cell lines, under a decree issued by President George W. Bush in August 2001. But that could be revoked at a stroke.

Fully debated federal legislation must be preferable to the current mish-mash. Britain's pioneering 1990 Human Fertilisation and Embryology Act has done a reasonable job of protecting the interests of both embryos and researchers. It's time to consider whether something similar might work in the United States. ■

Reform by stealth

The government of Silvio Berlusconi apparently wants to restructure Italian science, but seems uninterested in consultation.

These are worrying times for Italian researchers. Not only are they facing the prospect of budget cuts, but a proposal leaked from the research ministry in August has raised the spectre of further political control over the CNR, Italy's national research council.

The news that the right-leaning government of Prime Minister Silvio Berlusconi was planning to restructure Italian science came as a shock. After all, the point is not yet dry on reforms introduced by the previous centre-left government, which regrouped the CNR's 330 institutes into the hundred that now exist (see *Nature* **412**, 264–265; 2001). The leaked document's content was even more disturbing than its sudden appearance: it proposed dividing the CNR into 15 directorates, the head of each to be appointed by the research ministry, and suggested that a range of other independent agencies and institutes should be rolled into the CNR.

Italian science has long been plagued by a system that appoints the heads of research agencies according to political allegiance, rather than competence. And the prospect of this unwelcome patronage reaching further down into the management of the CNR, and encompassing bodies that currently lie outside the council's influence, has sparked petitions and rallies of protest (see *Nature* **419**, 240; 2002). Guido Possa, deputy minister for research, claimed the document was merely one of a number of very early drafts. But as the weeks have

passed with an absence of discernible consultation, few scientists are reassured that the final document, expected to be released later this month, will be significantly different.

So far, all that has emerged is a proposed 2.5% cut in the publicly funded research budget. What's more, the government proposes to end special funding arrangements for the INFN, an independent agency for research into atomic, molecular and condensed-matter physics created in 1994. As a result, the INFN faces having its core budget slashed by more than a third, while being subsumed into the CNR.

It is all a far cry from the first few months after the Berlusconi administration's election in April last year, when Possa's boss, education and research minister Letizia Moratti, committed to double the research budget over the next five years.

In line with Berlusconi's business-friendly ideology, Possa likes to compare research organizations to profit-making companies. This is misguided. Before it is too late, he should talk with, and listen to, the scientific community, in Italy and abroad, to explain what his government wants to achieve, and to learn how best to make use of his country's highly educated researchers. If he does so, Possa will learn that independence, not tight political control, is the key to success in basic research. He might also be reminded that reforms introduced alongside significant budget cuts are rarely a success. ■





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US biologists wary of move to view embryos as human beings

Erika Check, Washington

An air of unease has settled over US researchers who work on human embryos, following a significant shift in the remit of a committee that advises on research involving human subjects.

For the first time, the advisory committee is being asked to broaden its scope to consider the welfare of embryos and fetuses involved in research.

Some scientists suspect that the move is a step towards extending the legal protection given to human embryos and fetuses. Should such a revision win approval, it could hamper some kinds of research, such as work on embryonic stem cells.

The charter for the new Secretary's Advisory Committee on Human Research Protections, details of which were revealed in *The Washington Post* on 30 October, instructs its members to pay particular attention to "pregnant women, embryos and fetuses", among other groups. The panel was set up last month, although its members have

yet to be appointed. It is the successor to the National Human Research Protections Advisory Committee, set under the Clinton administration, which was not asked to consider the welfare of embryos or fetuses.

The committee is appointed by the Department of Health and Human Services (HHS), and does not have the power to make new rules. Instead, its recommendations must be passed into law by Congress or made into policy by HHS officials.

But many observers suspect that the Bush administration will use decisions made by the committee to back up the case for greater legal protection of embryos and fetuses. They are worried, for example, that health secretary Tommy Thompson may appoint committee members who support such changes to the law.

Stem-cell researcher Irving Weissman of Stanford University in California echoes these concerns. "I am very nervous that political activity at the input decides the outcome, and this administration may be



Ball of confusion: is an embryo a collection of cells or should it have the same rights as an adult?

Gene centre chips in with better route to microarrays

Alison Abbott, Munich

Biologists who make their own DNA microarrays, rather than relying on expensive commercial chips, should now be able to do a better do-it-yourself job, thanks to a set of human DNA clones made available by the German Genome Resource Centre (RZPD) in Berlin.

Microarrays are increasingly being used by researchers interested in gene expression, allowing them to compare the activity of thousands of genes in, for example, cancerous and non-cancerous tissues. Most commercial systems consist of arrays of thousands of short nucleotide synthetic sequences, called oligonucleotides, designed to bind to specific messenger RNAs (mRNAs), which are transcribed from active genes.

But many researchers opt instead to make their own cheaper microarrays from complementary DNAs (cDNAs), which are

made from mRNAs directly, using a reverse transcriptase enzyme. Each cDNA binds to the same mRNA from which it was created. Once a researcher has obtained a library of cDNAs, they can make copies of the cDNAs to create many microarrays.

The non-profit RZPD says that its human cDNA clone set is of much higher quality than those produced by other labs, and is also better catalogued. The RZPD's first customers say that they can use the cDNA clones to create their own microarrays at a fraction of the cost of commercial oligonucleotide arrays.

The RZPD set has several advantages over other libraries. Each clone has been resequenced and verified as corresponding to its database sequence. About 10–20% of samples in most other cDNA libraries are incorrectly labelled. The RZPD has also gone to great lengths to be as sure as possible that

each cDNA represents a unique gene, whereas most cDNA libraries contain multiple copies of mRNAs of particularly active genes.

"The availability of a sequence-verified, non-redundant cDNA library is fantastic — global approaches to biological research hinge on the availability of high-quality reagents," says Steven Gullans, co-director of the Gene Array Center at the Brigham and Women's Hospital in Boston.

The RZPD clone set contains 32,000 genes, and a further 10,000 will be added over the next few months — giving it more genes than other libraries. "Spotting 40,000 or so cDNAs onto a microarray means that we can cover a large part of the human genome," says Jobst Landgrebe, a biochemist at the University of Göttingen. He adds that it will cost him no more than 75 euros (US\$75) to make a microarray from the cDNA set. ■

♦ www.rzpd.de

going down that path," he says.

If the committee recommends that embryos should be afforded the same legal protection as adults, for example, researchers might not be able to use them in research that does not benefit the embryos. This could prevent researchers from using embryos left over from *in vitro* fertilization as sources of human embryonic stem cells.

Such a move would have implications for federally funded biomedical research, as the HHS has jurisdiction over the National Institutes of Health. It also oversees the Food and Drug Administration (FDA), and so can influence companies hoping to use research results to gain FDA approval for new drugs and therapies.

HHS spokesman Bill Hall denies that the change to the committee's remit is a political move, saying that it is directed at women who participate in studies, and not at scientists who do basic research. "It is incumbent on researchers to fully inform women who might be pregnant or might be planning to become pregnant about the risks of whatever study they're thinking about participating in," Hall says.

But some observers say that no matter what the intentions behind the change are, the move is still alarming for scientists and many other groups, such as those who wish to keep abortion legal. "It's a dangerous precedent to characterize a cluster of cells as a human subject," says Kevin Wilson, director of public policy for the American Society for Cell Biology. ■

Developing countries to gain from carbon-trading fund

Natasha McDowell

Rural communities in the world's poorest nations will be able to earn income by using their forests and agricultural land to sequester carbon dioxide, under a plan announced this week by the World Bank.

The BioCarbon Fund, launched on 5 November, will allow companies and public-sector organizations in the developed world to offset some of their carbon emissions by investing in projects in the developing world, such as tree-planting schemes, which absorb carbon from the atmosphere. Investors will earn credits that can be used to meet regulatory requirements or voluntary pledges to cut greenhouse-gas emissions.

The World Bank, which has set a target of US\$100 million for the fund, will control how the money is spent. It says that the fund will be supported by the private and public sectors. Fourteen businesses, ranging from power utilities to insurance companies, have already indicated an interest.

Ian Noble, a World Bank official who is chief adviser to the fund, says that investment will be made according to three criteria: cutting greenhouse-gas emissions, benefiting the environment by promoting biodiversity, and reducing poverty by encouraging sustainable development. One project already submitted for consideration involves planting a buffer of native trees around a Ugandan conservation area that has been encroached upon by local people. Locals will also be able



The White Nile flowing through Uganda, one of the nations that may benefit from the new plan.

to collect non-timber products from this buffer area.

Action groups have long advocated the use of carbon trading for social benefits. Last month, for example, the Centre for International Forestry Research in Bogor, Indonesia, and Forest Trends, a think-tank based in Washington DC, released a report showing that small-scale forestry projects integrated with rural agriculture can provide a cheap and comparatively low-risk way for poorer nations to generate carbon credits.

The BioCarbon Fund has earned further praise by including projects that are not covered by the Kyoto Protocol, the international agreement on limiting greenhouse-gas emissions. Conserving or restoring existing forest cannot be used to earn credits under the protocol, for example. But such projects will be covered by the new fund. ■

Academies back Smithsonian's calls for direct funds

Kendall Powell, Washington

Smithsonian Institution researchers breathed a collective sigh of relief last week, although their troubles may be far from over. Two reports released on 31 October state that the institution provides world-class science, and should continue to receive federal funding. But the future structure and funding of the Smithsonian depend on the outcome of a third report, expected in January.

The institution's research centres have been under a cloud for two years. Critics, including the White House Office of Management and Budget, suggested that Smithsonian scientists should compete for their funding rather than get it directly from government. Two of the centres were also threatened with closure, and a major reorganization seemed likely.

But last week's reports from the National Research Council, which is part of the

National Academies, and the independent National Academy of Public Administration (NAPA), will help to lift the gloom. The studies, which were commissioned by the Office of Management and Budget, say that removing direct funding could harm or even end top-notch research programmes.



Building on success: two reports say that the Smithsonian delivers world-class science.

"It's a great thing to have outside organizations recognize the contributions we make," says Paula DePriest, associate botany curator at the Smithsonian's National Museum of Natural History in Washington DC. "We're going to see an increase in the morale of scientists here."

According to the reports, the federal funding — \$111 million in 2002 — supports core functions such as researcher salaries and facility maintenance. Competitive grants and contracts already support the main thrust of research projects, and totalled \$98 million in 2001, according to the NAPA study.

The Smithsonian's researchers are hopeful that the reports will bolster their cause, but they still face an anxious wait. The institution's own Science Commission, which it set up to advise it on setting research priorities for the future, will deliver its final report in early January. ■

Theses spark twin dilemma for physicists

Declan Butler, Paris

Take a deep breath, and give the following sentence a go. "We demonstrate that the lorentzian signature of the space-time metric $(+++ -)$ is not fixed at the Planck scale and shows 'quantum fluctuation' between the lorentzian and euclidean $(+++ \pm)$ forms until the 0 scale where it becomes euclidean $(++++)$." Confused? Don't worry, you're in good company. Physicists around the world have been unable to agree on whether the PhD thesis this line comes from is good, bad or a hoax.

Rumours began to circulate after Max Niedermaier, a physicist at the University of Tours in France, sent an e-mail on 22 October to Ted Newman, a physicist at the University of Pittsburgh. Niedermaier alleged that French twins Grichka and Igor Bogdanoff, science writers who starred in a popular 1980s science television programme, had "spoofed" their PhD theses.

The line above comes from the abstract to Grichka's thesis, for which he gained his PhD at Bourgogne University in 2000. Both this thesis and that of his brother, who gained his PhD from Bourgogne earlier this year, drew on areas of mathematics and theoretical physics to study the origin of the Universe. Niedermaier claimed that the theses, and four of the brothers' papers published recently in peer-reviewed journals, had been concocted using jargon acquired from interviewing string theorists and other theoretical physicists.

"The abstracts are delightfully meaningless combinations of buzzwords ... which apparently have been taken seriously," Niedermaier wrote. The claim prompted a flurry of e-mails and Internet postings, with many physicists echoing Niedermaier's thoughts. After speaking to the Bogdanoffs, Niedermaier quickly issued an apology, acknowledging that he had not relied on "first hand information". But the message came too late.

With doubt hanging over the Bogdanoffs, physicists rushed to distance themselves from the twins' papers. The editorial board of *Classical and Quantum Gravity* took the unusual step of saying that a paper by the brothers had slipped through peer review "even though, in retrospect, it does not meet the standards expected of articles in this journal".

Frank Wilczek, editor-in-chief of the *Annals of Physics* and a theoretical physicist at the Massachusetts Institute of Technology (MIT), disowned another paper, arguing that it had been accepted by past management and would not have been accepted under him.

So are the papers good science or not? Enquiries by *Nature* show that few theoretical physicists, including some who reviewed the brothers' PhD theses, are completely certain. Jac Verbaarschot, of Stony Brook University in New York, reviewed Igor's PhD.



STRING THEORISTS DISCUSS KNOT THEORY WITH THE BOGDANOFF TWINS.

He says it contained original ideas, but claims that it was awarded in part because of Igor's contributions to the public understanding of science. Others have come to harsher conclusions. "They were at best wrong, and most likely just throwing around words with no calculations or proofs to back them up," says Lee Smolin of the Perimeter Institute for Theoretical Physics in Waterloo, Canada, who has studied some of the papers.

But Roman Jackiw, a physicist at MIT who reviewed Igor's thesis, insists that it is of the

requisite quality. Robert Coquereaux, from the International Centre for Mathematical Meetings in Marseille, has said that the brothers' work is certainly no better or worse than that of some established theoretical physicists.

The brothers, who are currently presenting a short television programme in France, insist that their work is genuine. They say that many critics haven't actually read their entire theses, which are available only in French, and that none of the criticisms made discredits their work. They also point to referees' reports on their theses. Verbaarschot, for example, declared that Igor's PhD "ranks as one of the best I have seen in recent years".

With no clear consensus emerging, the credibility of the peer-review system and journals in string theory and related areas is taking a battering. Peter Woit, a mathematician at Columbia University in New York, says that the incident illustrates the speculative nature of much theoretical physics. "The Bogdanoffs' work is significantly more incoherent than just about anything else being published," he says. "But the increasingly low standard of coherence in the whole field is what allowed them to think they were doing something sensible and to get it published." ■

Health initiative gets warm welcome

Geoff Brumfiel, Washington

Plans for a more integrated approach to the world's health problems are gathering momentum, according to their architect Richard Klausner, a former director of the US National Cancer Institute.

Klausner wants to increase the effectiveness of public-health campaigns in developing countries by merging areas such as economics and political science into public-health thinking. This field of 'global health sciences' would also foster connections between institutions in the developed and the developing world to bolster the public-health infrastructures of poorer nations.

During the six months since he became executive director of the global health programme at the Bill & Melinda Gates Foundation in Seattle, Washington, Klausner has met with researchers across Europe and the United States to advocate the creation of the new field. "The people I've spoken to are very intrigued," he says.

"It's time to change how we do public health," agrees Josh Ruxin of the Center for Global Health and Economic Development at Columbia University in New York. Poverty, corruption and regional instability



Seeking change: Richard Klausner.

are often critical factors that determine how an epidemic unfolds. "But you don't learn how to deal with those issues in public-health schools," Ruxin says.

Partnerships between the developed and developing world are also essential to improving public health, adds Amir Attaran, a public-policy expert at

Harvard's John F. Kennedy School of Government. "There are astonishingly few scientific resources in developing countries," he says. Fostering research communities through collaboration would go a long way towards increasing the quality of public health, he suggests.

Klausner, who oversees half of the roughly US\$1 billion in grants given out by the Gates Foundation, is optimistic that institutions will take up his idea. "Stay tuned," he says. "I think a lot is going to happen in the next year or so." ■

Mussel model calls for cash to combat invading species

Tony Reichhardt, Washington

Spend now to save later — that's the message from biologists Brian Leung and David Lodge, who have made what they say is the first attempt to merge ecology and economics into a mathematical model for analysing the risks from invasive species.

Leung and Lodge, who are based at the University of Notre Dame in Indiana, used zebra mussels as a test case. These molluscs cost US industry about \$100 million a year, primarily by clogging pipes and reducing water flow to lake-side power plants.

The researchers modelled the rate at which the invaders spread and weighed up the economic factors involved, such as the costs of preventing the mussels from invading new lakes. They calculate that it is worth spending up to \$324,000 a year to stop zebra mussels from contaminating a lake with a large power plant (B. Leung *et al. Proc. R. Soc. Lond. B*; in the press). As the US Fish and Wildlife Service spends just \$825,000 a year controlling all non-indigenous aquatic species in the country's lakes, Leung and Lodge conclude that "a much higher value should be placed on prevention".

Environmental economics is still a relatively new field, but quantitative tools such as this may help policy-makers to decide when it pays to prevent the spread of invasive species, says Lodge. "These sorts of analyses just haven't been done," he says, even though damage caused by invasive species costs the United States about \$137 billion a year.

With no similar studies available, it is unclear how widely Leung and Lodge's results can be applied. But their model suggests that, for zebra mussels at least, the problem should not be ignored. Over five years, it showed that it was cheaper to do nothing. But for 25 years, the economic benefits of prevention were clear. ■



Mollusc pollution: but is it worth shelling out to stop mussels from invading lakes?



Collision course: the KEK accelerator is now producing unprecedented numbers of B mesons.

Japanese physicists beam as meson production soars

David Cyranoski, Tokyo

The cases of empty beer bottles at the High Energy Accelerator Research Organization (KEK) in Tsukuba, Japan, are testimony to the celebrations held there in recent weeks.

KEK is competing with the Stanford Linear Accelerator Center (SLAC) in the United States to produce ever higher numbers of B mesons — subatomic particles that can be used to test the standard model of high-energy physics. On 2 October, KEK surpassed the number of B mesons produced at SLAC, before going on to hit the 100-million mark late last month. KEK researchers say that they are now leading the race to search for physics beyond the standard model.

Both accelerators bring electrons and positrons into high-energy collisions, producing B mesons and their antiparticle equivalent, anti-B mesons. KEK's B-meson factory is designed to generate collisions at a higher rate than SLAC's. But technical problems left it operating at a fraction of its capacity when it started taking readings in May 1999 (see *Nature* **403**, 586–587; 2000), allowing SLAC to generate more data.

The addition of solenoid magnets, made by winding wire around the metal that encases the beams, helped KEK to stabilize its electron and positron beams. Together with other improvements, this helped KEK to reach almost twice the rate of electron-positron collisions achieved at SLAC. The turnaround is a welcome reward for the scientists who attached the 9,000 solenoids by hand. "My hair turned grey while we were struggling with that," says KEK researcher Masakazu Yoshioka.

Both groups have already detected differences in the decay rates of the two types of B

meson, which could help to explain why there is now more matter than antimatter in the Universe. Physicists believe that the two types existed in equal amounts at the Big Bang. Studying differences in the behaviour of matter and antimatter could help to explain how matter came to dominate (see *Nature* **418**, 469; 2002).

Further data will help researchers to examine the decay rates in more detail, and could point to physics that contradicts the standard model. Kazuo Abe of the Belle collaboration at KEK, which analyses the B-meson data, says that at least 500 million, and maybe 2 billion, B mesons must be produced to search for such deviations. "The high levels of production are extremely important," he says. "If KEK continues to produce data at nearly twice the rate of SLAC over the next couple of years, there will be a big difference between what we can do and what SLAC can do."

But SLAC may not lag behind for long. Its design capacity is less than that of KEK, but it is already performing above its designed collision rate. Operations at SLAC are suspended until 15 November while physicists increase the accelerator's collision rate and upgrade the detectors. "When we come back, we expect to have the same performance as Belle," says Marcello Giorgi, a physicist and spokesman for BaBar, SLAC's B-meson detector. "Perhaps in the future we will have something better."

The competition may ultimately benefit everyone involved. "KEK passes SLAC, then SLAC passes KEK — this just continues on and on," says Burt Richter, a former director of SLAC who attended the celebrations at KEK. "Having two separate groups will make sure we get honest results." ■

Hostage deaths put gas weapons in spotlight

Quirin Schiermeier

Is there such a thing as a 'non-lethal' chemical weapon? The tragic end to the hostage crisis in Moscow, in which 117 hostages were fatally poisoned by a gas intended to incapacitate the hostage-takers, has propelled this question to the top of the anti-terrorist agenda.

Even now, the exact nature of the gas used by soldiers to free the 700 hostages, held in Moscow's Musical Theatre by Chechen gunmen with explosives strapped to themselves, is not entirely clear. Several days after the incident, Russian health minister Yuri Shevchenko said that the gas was an aerosol containing fentanyl, a potent and fast-acting opiate, which is used clinically as an anaesthetic and painkiller.

But fentanyl may have been only part of a mixture of gases used in the assault. Scientists at the Rechts der Isar University Hospital in Munich found traces of halothane, another general anaesthetic that is no longer widely used, in blood and tissue samples taken from two German hostages who survived the assault. "We are 100% certain that we identified halothane," says Thomas Zilker, head of toxicology at Rechts der Isar. He was unable to detect fentanyl in the samples, but as it is rapidly metabolized, he says, it would in any case no longer have been detectable in the two hostages when they arrived in Munich two days after the attack.

High risk

Using fast-acting anaesthetics outside the operating theatre is highly risky regardless of their composition, scientists say. Both fentanyl and halothane have very narrow 'therapeutic windows', meaning that potentially fatal side-effects, including respiratory depression, occur at doses only slightly higher than those required for their therapeutic effects.

"Halothane and fentanyl are lousy agents, both with terrible effects on humans," says Alan Zelicoff, a senior scientist at the Center for National Security and Arms Control at Sandia National Laboratory in Albuquerque, New Mexico. "It was a grotesque assumption on the part of the Russian leadership that sloppy use of highly effective anaesthetics, pumped into a confined room full of weakened hostages, would not kill many people."

But under the circumstances of the crisis, Russia may not have had many alternatives, Zelicoff concedes. "I can think of no fast-acting agent that could be safely used as a knockout gas in such a situation," he admits.

The US Defense Department's Joint Non-Lethal Weapons Program supports an



Liberated hostages are driven from the scene of Moscow's siege, in which 117 hostages were killed in a gas attack by Russian forces. Survivors were found to have inhaled potent anaesthetic compounds.

extensive drug-based weapons programme for law-enforcement purposes. At Pennsylvania State University's Institute for Emerging Defense Technologies, for example, researchers are investigating the potential of 'calmatives' based on benzodiazepines, a class of compounds that includes anti-anxiety drugs such as Valium. Such agents have wider therapeutic windows, so would be safer, says Zelicoff, but they would not have been fast-acting enough to have been useful in the Moscow situation.

Banned substance?

Non-lethal chemical weapons for law-enforcement purposes are allowed under the Chemical Weapons Convention (CWC). The CWC treaty, which bans the use of toxic chemicals for all military purposes, was ratified by Russia and the United States in 1997. The jury is still out on whether Russia violated the CWC in using chemical weapons against the Chechen gunmen — Russia's ongoing conflict with Chechnya has led to the assault being interpreted by many as military rather than civilian. "We are awaiting official information from the Russian federation," says a spokesman for the CWC Secretariat in The Hague.

But given the increasingly indistinct boundaries between military and civilian

purposes, and the unavoidable risks associated with the mass distribution of any chemical, critics oppose the development and use of chemical agents in any circumstances.

"After Moscow, we have to rethink the medical implications of all 'non-lethal' agents, given the impossibility of getting an appropriate dose to all individuals in a large crowd," says Jan van Aken, a toxicologist who is head of the Hamburg-based office of the Sunshine Project, a US-German pressure group that deals with issues of biological and chemical warfare.

Toxic toll

Van Aken even challenges Zelicoff's assumption that calmatives would be safe. "Toxicologists estimate that even substances with a wide therapeutic window such as Valium would cause around 5% casualties if used in concentrations high enough to knock out kidnappers," he says.

Zelicoff admits that non-lethal chemical weapons are a contentious issue. But outside the battlefield, chemical agents provide a humane alternative to conventional weapons, he argues. "It always comes down to the intent, of course," he says. "But please imagine a shot-down pilot surrounded by non-combatants: would it be more humane to disperse the crowd with tear-gas or with hot lead?"

D. LOVETSKY/AP PHOTO

Controversial HIV study puts whole-cat work on pause

Columbus, Ohio Researchers using a controversial cat model of AIDS in an attempt to understand how the HIV virus causes brain damage in drug abusers will continue their project. But the project, based at Ohio State University, will not use whole animals for the time being.

The project's future became uncertain when its principal investigator, Michael Podell, left the university after animal-rights groups protested against his work (see *Nature* **417**, 778; 2002). Podell's model involved injecting cats with the HIV-like feline immunodeficiency virus.

Lawrence Mathes, director of Ohio State's Center for Retrovirus Research, has now taken over the \$1.63-million project, which is funded by the National Institute on Drug Abuse. Mathes will follow up Podell's *in vitro* finding that the virus replicated more efficiently in cultured brain cells when the cells were also treated with the recreational drug methamphetamine.

But he denies that his decision to stop whole-cat research was a result of pressure from activists. "We found an underlying mechanism that may be important for the

neurological form of the disease and we've modified the specific aims of the grant," he says. Whole-cat studies may be needed in the future to verify *in vitro* work, he adds.

Cassini sends first postcard from long-haul trip to Saturn

Washington A camera on board the interplanetary spacecraft Cassini has sent back its first colour image of Saturn, its final destination. The picture was taken from a distance of 285 million kilometres, nearly twice the distance between the Earth and the Sun.

The picture shows Saturn's southern hemisphere, and the shadow Saturn casts on its own rings. "This is an emotional event for the mission," says Dennis Matson, a Cassini project scientist at NASA's Jet Propulsion



Ring of confidence: this image of Saturn should be the first of many to be produced by Cassini.

Laboratory in Pasadena, California. "We now have Saturn in our sights."

After a seven-year voyage, Cassini will enter into orbit around Saturn in July 2004, to begin a four-year study of the gas giant and its 30 known moons. The project includes the release of the Huygens probe, which is scheduled to descend into the atmosphere of Saturn's largest moon, Titan, in January 2005. ♦ <http://saturn.jpl.nasa.gov>

Single-cell DNA trap set to finger criminals

Brisbane It's a bad time to be a criminal. Australian scientists say that they can now identify an individual on the basis of a single cell left on materials at the scene of a crime — with a ten-billion-to-one certainty.

Ian Findlay of the Australian Genome Research Facility at the University of Queensland, Brisbane, and colleagues have achieved a 100-fold improvement in the accuracy of the single-cell DNA-fingerprinting technology that they developed five years ago (I. Findlay *et al.* *Nature* **389**, 555–556; 1997). And they can now analyse thousands of cells each day.

The technology, which Findlay reported last week at the 'Spy vs Spy' meeting at the University of Technology in Sydney, has many potential security applications. These

include authenticating documents and tracing the hands through which laundered money has passed. The technology could also help police to identify individuals from biological samples containing mixtures of cells, such as in cases of gang rape.

One small step by NASA aims to end hoax lunacy

Washington Thirty years after the last Apollo astronauts landed on the Moon, a few stubborn conspiracy theorists continue to insist that the whole thing was faked.

NASA has finally had enough. It has commissioned space writer and veteran UFO debunker James Oberg to write a 30,000-word monograph refuting the notion that the Apollo programme was a hoax. Oberg says that the target audience will not be the conspiracy-mongers themselves, but members of the public confused by programmes such as a television special, aired by the US television channel FOX last year, that gave the hoax theory credibility.

NASA's own attempts to convince the doubters have been ineffective, says Oberg. He intends to counter the arguments of the hoax theorists by pointing out factual errors in their claims that can be checked by the public. "I'm looking to communicate, not pontificate," he says.

Nuclear clean-up hope mounts at summit

Bishkek A pledge to clean up nuclear waste dumps that threaten to contaminate one of central Asia's most densely populated areas has emerged from the first ever Global Mountain Summit.

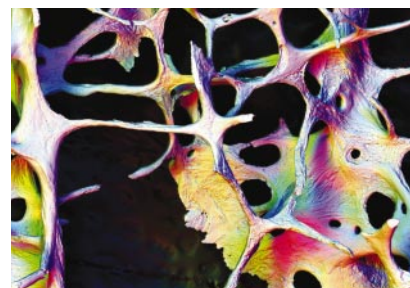
At the close of the summit, held last week in Bishkek, Kyrgyzstan, Norwegian delegates offered to help make the dumps safe. The waste is stored in deteriorating dams close to the town of Maily-Suu in the mountains of Kyrgyzstan. It threatens to spill into rivers that flow into the Fergana valley, home to almost 20% of central Asia's population.

Delegates at the summit, the final event of the United Nations International Year of Mountains, also agreed on the need to safeguard mountain environments and communities from threats such as climate change, which they hope will help guide national policies.

♦ www.globalmountainsummit.org

Gallery gasps as science makes an exhibition of itself

London Is artifice and manipulation in scientific image-making a form of art? This is the question being framed at the Wellcome Trust's new exhibition 'Truth and Beauty' at



But is it art? The first fully focused scanning electron microscope image goes on show.

the TwoTen gallery in London. The exhibition includes work from contemporary artists inspired by science, alongside works of the 24 winners of the 2002 Biomedical Image Award. The winning images were selected from those acquired by the Wellcome Trust's Medical Photographic Library during the past year.

The picture above, for example, produced by Alan Boyde, emeritus professor at University College London's Department of Anatomy and Developmental Biology, shows damage to a vertebral bone in osteoporosis, and is the world's first fully in focus scanning electron microscope image. The effect is achieved by creating a single image from many different individually focused images.

The exhibition runs from 8 November 2002 to 21 March 2003.

Out of this world

NASA's Institute for Advanced Concepts aims to turn speculative ideas into tomorrow's space missions. Tony Reichhardt attends its latest get-together, and asks whether the investment is worth it.

Fact or fiction? An early version of an elevator to send people into space, one unconventional idea being explored by NASA's advanced concepts institute.

For an organization that declares itself “bounded only by the limits of the human imagination”, NASA's Institute for Advanced Concepts (NIAC) is housed in a surprisingly down-at-heel neighbourhood. Home is a nondescript, grey building in a slightly run-down section of Atlanta. Looming a mile or so in the distance are the glittering high-rise offices of the South's largest city, but this part of town is all ‘space for rent’ signs and cracked pavements.

Out of such surroundings, NASA hopes to nurture some big ideas. Established in 1998 with an annual budget of about \$4 million, NIAC funds researchers from outside NASA who want to work on long-term, speculative projects. “We’re not looking for evolutionary ideas,” says director Robert Cassanova, an aerospace engineer who used to run the nearby Georgia Tech Research Institute. “We’re looking for great leaps forward.”

To date, more than 75 researchers have answered his call. Some propose technology that appears to be achievable, such as sails that use sunlight to propel spacecraft. Plans to send people and cargo into Earth orbits using space elevators are more speculative. A few ideas, such as a study of a propulsion system based on a hypothesized form of matter known as the hydrino, are deemed unworthy of study by most researchers. But all fall within NIAC's remit: the exploration of ideas that are likely to require 10–40 years to bring to fruition.

Late last month, recipients of NIAC funding gathered at a two-day workshop at the

institute's headquarters to discuss their progress. “Don’t let your preoccupation with reality stifle your imagination,” declared one of Cassanova's slides, and first-time attendees could be heard introducing themselves with: “So what's your crazy idea?” But this was no crackpot convention, and there wasn't a warp drive in sight.

The outer limits

Cassanova says that a “good handful” of the 600-plus proposals received by NIAC to date have been science fiction. But these don't qualify for phase I funds — the institute's initial six-month grants of up to \$75,000. NIAC fellows are, for the most part, from universities, government labs and small aerospace companies. Meeting presentations resemble those at other NASA technical gatherings, with the main difference being that speculation is encouraged. Audience members may ask for clarification, but if anyone has doubts about whether an idea is practical, or even possible, they keep them to themselves.

After two days of presentations, the difficulty of running a grants programme for space missions that are decades away becomes clear. NIAC has a radical philosophy, but other institutes have far greater resources. With its limited budget, what can NIAC contribute?

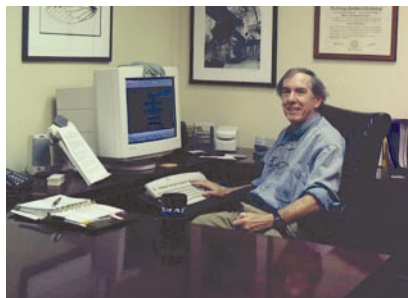
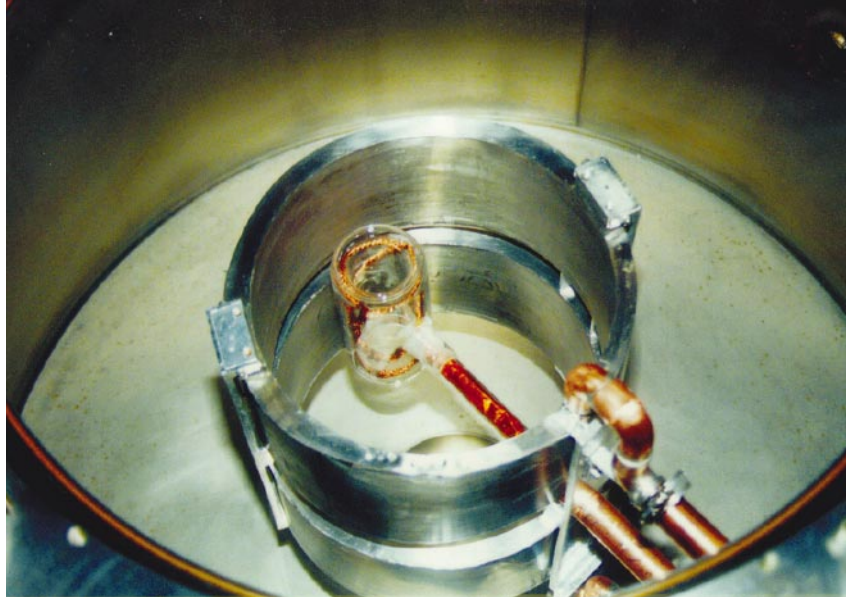
Often it is a matter of finding a use for cutting-edge research from other fields. Steven Howe, a physicist formerly with the Los Alamos National Laboratory in New Mexico, has a

phase I grant to develop plans for an antimatter propulsion system. Antimatter rockets, which would use the energy produced when matter and antimatter annihilate each other, have been dreamed about for decades. But Howe, who founded Hbar Technologies, a Chicago-based company that aims to develop antimatter technologies, points out that physics is now catching up with science fiction.

Last month, researchers at CERN, the European particle-physics laboratory near Geneva, described the production of about 50,000 antihydrogen atoms — the first time such a large quantity has been generated (M. Amoretti *et al.* *Nature* **419**, 456–459; 2002). And Fermilab, near Chicago, Illinois, is producing upwards of 10^{14} antiprotons a year. Howe says it should soon be possible to make the 30 milligrams or so of antimatter needed to send a spacecraft to the Kuiper belt, the band of rocks and ice beyond Neptune.

Howe's design fires an antihydrogen beam at the uranium-coated inner surface of a sail to trigger a fission reaction. He hopes to secure phase II funding, which can provide up to \$500,000 over two years, to investigate how much momentum this reaction would transfer to the sail, and to study the antimatter storage techniques being developed at CERN and other labs.

Other NIAC fellows want to exploit existing technology. Anthony Colozza at the Ohio Aerospace Institute in Cleveland has his eyes on the ‘entomopter’, a flying robotic vehicle that mimics the motion of an insect's wings.



Thrusting forwards: the test set-up for a plasma-induced magnetic bubble (top), and the insect-like Mars entomopter — two of the concepts being backed by Robert Cassanova and his institute.

The device is the work of Robert Michelson, an engineer at the Georgia Tech Research Institute, who developed it for the US Department of Defense. Colozza has designed a system for surveying the surface of Mars using a camera carried by the entomopter. The device is ideal for use on the red planet, where the thin air would make it impossible for craft with conventional wings to fly at low speeds, or to land. NIAC liked the idea enough to give it phase II funding.

Then there are the genuinely novel ideas. In the first round of NIAC grants in 1998, Robert Winglee, a space physicist at the University of Washington in Seattle, won funding for his Mini-Magnetospheric Plasma Propulsion (M^2P^2) concept. Winglee proposes creating a large magnetic bubble around a spacecraft by using a plasma to expand an existing magnetic field. The bubble would deflect the stream of charged particles, known as the solar wind, that flows from the Sun.

The small but constant pressure against the bubble's large surface area would accelerate a 200-kilogram payload to 80 kilometres per second within three months. A trip to Pluto would take six years, instead of the ten it takes now. Better yet, building such a vehicle would not require huge technological leaps.

The M^2P^2 study was an eye-opener for NASA, admits Murray Hirschbein, who is senior adviser to the agency's chief technologist. "Instead of something 10 to 40 years in the future, it may be a lot closer," he says.

Winglee's NIAC grant expired last year, but the work has already produced promising results in test chambers. It is likely to be picked up by NASA's advanced propulsion programme, and the agency might produce a working prototype for testing in space.

Mission improbable

Not all NIAC fellows are so lucky. The institute aims to draw clever ideas from outside NASA, validate the best ones and have the space agency take over. But some researchers say that "not invented here" attitudes within NASA ensure that few NIAC ideas will ever do more than gather dust.

One such critic is Ivan Bekey, a space technology consultant from Annandale, Virginia, and former head of NASA's advanced concepts programme, an in-house scheme that pre-dates NIAC. After leaving the agency in 1997, he won a phase I NIAC grant to study the feasibility of a large space telescope with no supporting structure, just a thin membrane shaped into a reflecting surface. The telescope's instruments would fly in formation to collect radiation reflected by the membrane. If the technique worked, it would lead to revolutionary devices 125 times lighter than the Hubble Space Telescope, but with mirrors ten times the diameter of Hubble's.

Bekey was turned down for a phase II grant but received funding from the National Reconnaissance Office, a government body that builds spy satellites. He says that an

advanced concepts programme would be better off run from NASA's Washington headquarters. Bekey doesn't dispute the quality of NIAC's work, but says that it is divorced from the rest of NASA. Without advocates within the agency, NIAC is doomed to generate "nice reports, and that's it", he says.

Success stories such as Winglee's plasma propulsion do indeed appear rare, and most phase II concepts end up as orphans. The entomopter, for example, is too immature a technology to be incorporated into the Mars missions planned for the next decade, which leaves Colozza struggling to find funding to continue his work now that his phase II funding has ended.

But Hirschbein isn't worried if most NIAC ideas languish on the shelf. "These are advanced concepts," he says. "They're not going to find a direct home right away." NIAC's real purpose, he adds, is to influence NASA's "general thought process". That means the NIAC fellows may not even be involved if their idea eventually flies.

But what of NIAC ideas that only a tiny minority believe will ever take off? Projects that are, in Cassanova's words, "on the fringe". Exhibit A is the BlackLight Rocket. According to its backers, the device uses a mysterious new form of power generated when a hydrogen atom becomes a hydrino — a hydrogen atom whose electron is unusually close to its nucleus.

Mainstream physicists contend that electrons cannot approach the nucleus as closely as the hydrino team predicts. But Anthony Marchese, a mechanical engineer at Rowan University in Glassboro, New Jersey, has a phase I grant to study the propulsion possibilities of such an energy source. Marchese doesn't buy all the claims being made by BlackLight Power, the New Jersey company that is developing hydrino-based technologies, but he does believe that "there is something going on that's interesting".

This vagueness, together with the ridicule heaped on hydrino research by figures such as Robert Park, the American Physical Society's director of public information and a keen observer of pseudo-science, makes some NIAC officials nervous about having funded Marchese's work.

But others say this twilight zone between safe and wacky is where NIAC belongs. During a break in the workshop, engineer Donna Shirley, who worked on NASA's celebrated Mars Pathfinder mission in the 1990s before moving to the University of Oklahoma, points out that many of the ideas could be duds, but the investment is worthwhile if an occasional jewel is produced. And the ideas presented at this meeting, says Shirley, are more interesting than the fare at other advanced concepts workshops. But, she adds with a smile, "not as good as at a science fiction convention". ■

Tony Reichhardt writes for *Nature* from Washington.

► www.niac.usra.edu

Put your lab in a different class

In many countries, students are turning away from the hard sciences. Can initiatives that give young people hands-on experience of research help to lure them back? Sally Goodman goes back to school.

When the US House of Representatives Committee on Government Reform met in February to hear evidence on anthrax research, its members were handed small plastic models of one of the anthrax toxins to help explain how drugs could be developed to treat infection with the bacterium. Little did they know that this physical representation of the protein had been designed by high-school students, using crystal-structure coordinates provided by the biologists giving evidence that day.

The project that yielded these models is just one of several schemes around the world that aim to involve teenagers in cutting-edge research. Their goal is to communicate the excitement felt by working scientists to school students who might otherwise perceive science as dry and dusty—and so to make young and enquiring minds consider taking a science degree, rather than opting for media studies or some other trendy subject. And if you've ever thought that you wanted to get involved in school science education but were unsure of what you could contribute, these schemes might provide some pointers.

The Team Anthrax project is a good place to start. Five months before the House committee hearing, in October 2001, mailed anthrax attacks were spreading terror across the United States. At that time, three students at Riverside University High School in Milwaukee, Wisconsin, were discussing biomolecular structures with their teacher, Jeff Anderson. Their goal was to turn such structures into three-dimensional models at the Milwaukee School of Engineering (MSOE). With anthrax dominating the news, its toxins were an obvious choice.

The MSOE's Center for BioMolecular Modeling (CBM) has the technology to turn structural data rapidly into plastic models. And Anderson had previously used the technology as part of a summer course for high-school science teachers. Back at his school, he realized that it could also provide the perfect means to get his high-school students excited about research.

Along with Tim Herman, the CBM's director, Anderson recruited a team of 17-year-olds committed enough to spend their early mornings, evenings and weekends working on the project. "It was like finding pieces to a puzzle," says Justin Snowden, one of the three student members of Team Anthrax.



Building success: molecular modeller Tim Herman (left) gets creative with Justin Snowden, one of the high school students involved in Team Anthrax, a project to create models of the pathogen's toxins.

"We found out that three proteins were involved in anthrax pathogenesis and then tried to find out who was working on them."

They first approached Wei-Jen Tang, a biologist at the University of Chicago who released his data on the structure of one of the toxins — the anthrax oedema factor — in advance of the publication of a paper describing the work (C. L. Drum *et al. Nature* **415**, 396–402; 2002).

Model students

Using this privileged information, along with data made available by researchers working on the other anthrax toxins, the students first produced computer images using a molecular visualization program. This was then interpreted by the machines at the MSOE's Rapid Prototyping Center to produce three-dimensional colour models of the three anthrax toxins. "It was a completely new way of thinking for us, as we continually refined our designs," says Mia DeFino, another Team Anthrax member.

Tang later visited the students. "It was a really rewarding exchange. I answered their questions on the protein's function and in return I got a wonderful model," says Tang, who says that he finds it useful as a communication

tool when discussing his work with colleagues.

Tang also suggested ways in which the students could refine the model to better highlight the protein's function. "When Professor Tang looked at the model he said it was like holding his dream," says Snowden. "That was a really important moment for us."

The students also visited the laboratory of John Young at the University of Wisconsin-Madison, who has studied the binding between one of the anthrax toxins and a receptor found on the surface of mammalian cells. "I was very impressed that the students had come up with this model. It is an excellent tool for testing hypotheses," he says. So impressed was Young, in fact, that he later ordered 24 small copies of the model when he was called to give evidence to the Committee on Government Reform.

For the students, meeting the researchers provided a real confidence boost. "I was flattered that they talked to me as if I was a graduate student — even if I didn't always understand what they were saying," says DeFino.

Team Anthrax also roused interest among their high-school peers. "Sixty-five fellow students turned up on a Saturday morning to listen to them," says Anderson. "I don't think it was just the chips and soda that



Matthias Eisel (above), one of 22 schoolchildren based at the Alfred Wegener Institute, gets to grips with ecology; left, Indiana students build components for high-energy physics studies.

brought them in!" He adds that the experience has also expanded his outlook as a teacher. "This has really raised my expectations of my students," says Anderson, who is now working with another group on the toxins produced by *Pseudomonas* bacteria.

Herman now plans to extend the experience across the United States, and is setting up six local centres where teams of high-school children will create similar models of different molecular structures in conjunction with local labs. "This approach levels the playing field between researchers and students. The students can offer something of real value to the researcher," says Herman.

The same philosophy of creating a meaningful partnership between students, teachers and researchers lies behind another US project, this time in high-energy physics.

In December 1997, when the United States joined the project to build the Large Hadron Collider (LHC) at CERN, the European particle physics laboratory near Geneva, Neal Lane, then director of the National Science Foundation (NSF), said: "The LHC is unquestionably science at the frontier, and the potential educational benefit is just as exciting and unprecedented." His comments prompted the NSF and the US Department

of Energy to sponsor an effort to create an educational outreach programme.

The result was QuarkNet, which now comprises 44 centres across the United States where physics teachers meet regularly with high-energy physicists at local universities. Some of the teachers will previously have worked in the physicists' labs over the summer break — programming software, entering data or testing equipment. The following summer, these 'lead' teachers co-opt another dozen teachers and run a workshop based on their work. Back at school, the teachers can give lessons about contemporary projects instead of textbook examples of nineteenth-century physics.

Labour of learning

But Randy Ruchti of the University of Notre Dame in Indiana, a physicist working on the Compact Muon Solenoid (CMS) experiment for the LHC, wanted to take the experience further and get students actively engaged. With the teachers involved in the Notre Dame QuarkNet centre, Ruchti converted an old pizzeria on the university campus into a lab. For the past three summers it has provided space for some 45 high-school students, aged 16 and 17, to come in and get paid for building components for Ruchti's experiments.

Last summer, the students built optical decoding units for use in the CMS. This involved threading optical fibres through holes in a box to ensure that the data would arrive at the right place when they were channelled through the other end. "This is very precise work, so it's good for young people with sharp eyesight," says Ruchti. Surprisingly, most of the students were not yet studying physics. "We get them green and unbiased, train them on the job and do the theory at lunchtime," says Ruchti.

"The students that came in to work with Randy saw that he had a real life and could converse normally," jokes Beth Beiersdorf, one of the teachers involved with Notre Dame's QuarkNet centre. She says that the experience was valuable both for academic high-fliers who might contemplate a career in research, and for those students with more practical skills. "They saw that there were also technicians handling the components as well."

Back at school, the effects are tangible. One teacher says that students who had worked with Ruchti started challenging him with probing questions. This shocked the other students until they saw that he responded positively, and then they joined in too.

Also through QuarkNet, physicist Yasar Onel of Iowa State University worked over the past summer with teacher Pete Bruecken and three of his students, testing photomultiplier tubes for the CMS. For Onel, the personal interactions were as important as the physics that the students learned. "They saw how we solved our problems," he says. "They became fully integrated and saw how important it was to work as a team."

The students produced a database of about 25 parameters to create a profile of the performance of 800 photomultiplier tubes which will be used over a 10-year period. When the CMS begins to produce data in 2007, says Bruecken, "it is possible that these same high-school students could use the data collected to do their thesis work in graduate school".

Similar initiatives are springing up in Europe. Indeed, one German research institute has invited an entire class of local high-school students to work alongside its researchers. "When we went to propose the idea to the schools we were quite sure they would say it was impossible," says Rainer Paulenz, administrative manager of the Alfred Wegener Institute for Polar and Marine Research (AWI) in Bremerhaven. "So when they replied that it would be no problem, we were astonished."

After only eight months of planning, the first 22 students, aged 15–17, arrived at the centre in August (see *Nature* **418**, 714; 2002). The students will spend two days a week at the centre over a three-year period and will be taught by their own teachers alongside AWI researchers.

In the first year, the students are working on special projects that link the school cur-

riculum in chemistry, biology and mathematics to the work of the institute, for example by calculating nutrient budgets in the Wadden Sea ecosystem. AWI researchers explain the techniques at the beginning of the project and then will get involved at the end to help to interpret the results. An English teacher has also been pressed into service, helping the students to understand information from English-language Internet sites.

AWI researchers were asked to produce a list of what they could offer to the project, ranging from short seminars on their research to assistance in identifying plankton. Trips on one of the institute's research vessels or to one of its island field stations will also be arranged for small groups of students. And so that these lucky few can share their experience — and data — with the other students, laptops have been provided so that all participants have access to an intranet dedicated to the project.

Once students have learned the basic skills in their first year, they will become involved in ongoing research at the institute. Ulrich Bathmann, an oceanographer at the AWI who helped to set up the scheme, says that researchers are currently looking at areas where students can contribute. He says, for example, that they could work on projects to document genetic diversity in phytoplankton.

Although the main motivation is educational, Paulenz also sees it as a way to develop researchers' ability to communicate their own work. "It will be interesting to see if people find other secondary benefits," he says.

In Britain, meanwhile, some students working towards A-levels or equivalent qualifications — the exams on which university entrance depends — can get involved in research through a bursary scheme run by the Nuffield Foundation, a charity with broad interests that include science education.

Selected students can choose a project from a list circulated by regional coordinators after researchers in universities and industry have been canvassed for their offers. Each researcher then gets to choose the student that they will take on, usually after a telephone interview. The scheme, which has been running since 1995, finances some 600 bursaries per year, and is today also backed by government-funded research councils and other charities, such as the Wellcome Trust.

Students work on their projects for four to six weeks over the summer holidays. "We do most of our research over the summer, so the students come into a really active lab," says Dominic Campopiano, a biochemist at the University of Edinburgh. Last year his Nuffield student spent six weeks in the lab and was able to clone a gene and overexpress a protein from *Aquifex aeolicus*, a bacterium that lives in scalding waters near volcanic vents on the sea floor. This student is the second author on a paper that Campopiano has recently submitted for publication. "He picked up the techniques quickly," says Campopiano.



Work done on snails by Mairi Struthers and her mentor Chris Viney could have medical applications.

For some students, the main benefit of the scheme comes not from opportunities to publish science but in helping to weigh up their career options.

Another student, Mairi Struthers, who two summers ago worked with Chris Viney, a chemist at Heriot-Watt University in Edinburgh, has already had a paper published (M. Struthers, G. Rosair, J. Buckman & C. Viney *J. Molluscan Stud.* **68**, 165–171; 2002). She worked on the biomineralization of the cement-like coatings that giant African land snails can secrete to seal their shells, generating findings that may have implications for mending broken bones. "It's important to choose a project that is going somewhere interesting," advises Viney. "Based on Mairi's findings I will now develop this area of research — it's an excellent springboard for larger projects."

For some students, though, the main benefit of the scheme comes not from early publishing opportunities but in helping to weigh up their career options. Laura Dalton had always wanted to be a vet but didn't know whether she would get the necessary exam grades. Her Nuffield bursary placed her with Clive Phillips at the University of Cambridge's Department of Clinical Veterinary Medicine, where she worked on a project on rabbits' feeding preferences. "I realized I didn't have to be a vet to work with animals and that I could do

animal-welfare research, for example, instead. It really took the pressure off," she says.

The Nuffield scheme seems so successful in generating enthusiasm that some researchers fear that its participants may be brought down to Earth with a bump when faced with the realities of undergraduate education. "You have to explain that what they are doing is cutting-edge research and they will have to go way back to the start and learn the fundamentals when they are undergraduates," says Campopiano.

Nevertheless, the students involved in these schemes are, most observers say, a fortunate few. And the researchers who have worked with them also consider themselves lucky. "It was incredibly exhilarating to work with such motivated and enthusiastic young people," says Ruth Robinson, a sedimentologist at the University of St Andrews, UK, who has had two Nuffield students in her lab.

The real test of these programmes will be whether they can be expanded to reverse the current trend in many industrialized nations towards declining enrolment in university science courses. Who knows, perhaps the House of Representatives Committee on Government Reform might one day include a scientifically enlightened member with a distant memory of a summer spent getting to grips with biomolecular structures. ■

Sally Goodman was until recently Nature's French correspondent.

Team Anthrax

♦ www.rpc.msoe.edu/sepa/teamanthrax.htm
QuarkNet

♦ quarknet.fnal.gov

Alfred Wegener Institute School Project

♦ www.awi-bremerhaven.de/AWI/Presse/PM/020916Schulprojekt-e.html

Nuffield Science Bursaries for Schools and Colleges

♦ www.nuffieldfoundation.org/grants/scibsc/overview.asp

Slow-moving journals hinder conservation efforts

Critical policy decisions miss out on research stuck in an 18-month publishing queue.

Sir—Conservation biology is a crisis discipline, burdened with the responsibility of providing rapid scientific answers that can help us protect our world's threatened biodiversity¹. Because we lack basic natural-history information regarding thousands of species on the precipice of extinction, there is a clear opportunity for biological research to make valuable 'real world' contributions. Indeed, conservation science can play a timely and pivotal role in judicial and legal decisions about resource policy².

However, to make a meaningful contribution to conservation decisions, scientific input is often needed on the timescale of months, rather than the years typical of many major scientific journals. Because of the urgency surrounding conservation, we investigated the speed with which the major conservation-biology journals publish research relative to comparable journals in organismal biology. Our results are disquieting. Rather than being faster, in response to the biodiversity crisis, leading journals in conservation biology are the slowest to publish primary research.

To discover how long it takes an original scientific finding to find its way into the peer-reviewed literature, we examined a wide array of biological journals and recorded the time in days from submission to publication for every research paper appearing in 2000. We excluded journals that appear more often than bi-weekly or less often than quarterly, or that fail to list the dates on which manuscripts were both received and accepted, and we focused on research articles (excluding special features, commentaries and essays). We selected journals to represent four categories: conservation and applied ecology; taxonomically oriented research; behaviour; and evolution and genetics. Within each category, we selected journals that have the highest science-impact scores³ or that represent the largest professional scientific societies. Journals satisfying both criteria were chosen first.

Among the examined journals, the three conservation and applied-ecology journals stand out as having the slowest publication processes (Fig. 1), with an average median lag time of 572.2 days from submission to publication. In contrast, the four genetics and evolution journals had an average median lag time of only 249.1 days. It is noteworthy that three of the four journals in this 'fastest class' encourage or require online submission, whereas none of the conservation journals allows online submission.

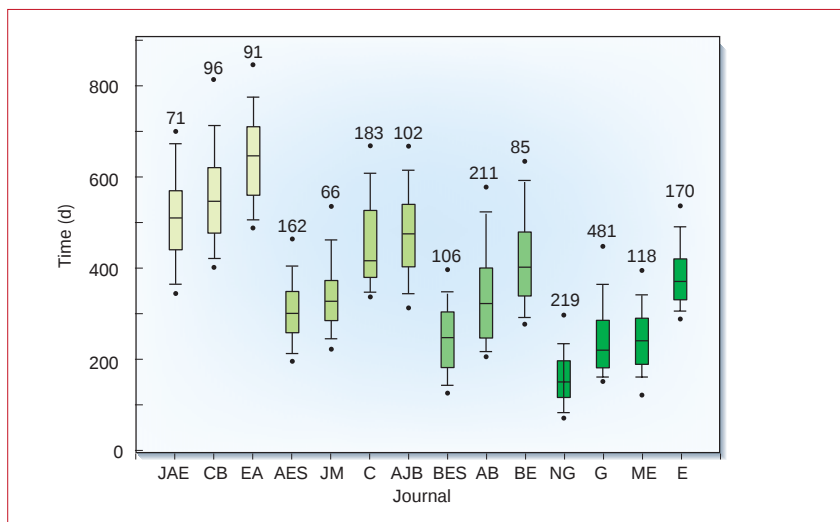


Figure 1 Median time, in days, from submission to publication for journals representing four disciplines within organismal biology. Conservation and applied ecology (yellow columns): JAE, *Journal of Applied Ecology*; CB, *Conservation Biology*; EA, *Ecological Applications*. Taxonomic (light green): AES, *Annals of the Entomological Society of America*; JM, *Journal of Mammalogy*; C, *Condor*; AJB, *American Journal of Botany*. Behaviour (medium green): BES, *Behavioral Ecology and Sociobiology*; AB, *Animal Behaviour*; BE, *Behavioral Ecology*. Evolution and genetics (dark green): NG, *Nature Genetics*; G, *Genetics*; ME, *Molecular Evolution*; E, *Evolution*. Extent of the boxes indicates 25th and 75th percentiles, lines within boxes represent medians, capped bars represent 10th and 90th percentiles, and circles represent 5th and 95th percentiles. Numerical labels are sample sizes.

We believe that the leading journals in the area of conservation biology have a heightened responsibility for rapid dissemination of research results. Our survey indicates that these scientific outlets are not measuring up to this responsibility. If conservation biology truly is a crisis discipline, then the discipline's journals must make rapid handling of submitted research articles a high priority. Otherwise, if we cannot rapidly publish science concerning threats to biodiversity,

opportunities for conservation action could be missed and species may pay the price for our procrastination.

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1. Wilson, E. O. *Conserv. Biol.* **14**, 1 (2000).
2. Harrison, S., Stahl, A. & Doak, D. *Conserv. Biol.* **7**, 950 (1993).
3. ISI Journal Citation Reports (Institute for Scientific Information, Philadelphia, 2000).

Sharp eyes saw through early effort to fake prints

Sir—While surveying the history of fingerprinting at the National Archives of India in New Delhi, we came across an early reference to forged fingerprints, similar to that described in your fascinating News item "Detectors licked by gummy fingers" (*Nature* **417**, 676; 2002; and see D. Ehrenfeld, *Nature* **418**, 583; 2002).

In 1917, when the science of fingerprinting was in its infancy, news of a demonstration by a lawyer in a court in Howrah (Bengal, India) threatened to undermine the value of this discipline

(*Home Department Proceedings* 202–206 (A), Police Branch, Government of India, August 1919).

The lawyer, Babu Panchkowsy Chatterji, was then invited to Bengal Fingerprint Bureau and asked to re-demonstrate his experiment. He took a thin piece of paper and smeared it lightly with gum arabic. He then placed it over a fingermark and pressed it for one to two minutes. Next, he slightly wetted the paper and separated it from the original impression so that it now carried the negative of the imprint. He applied the negative to a fresh sample of paper, wetted and pressed it again, and removed it, thus producing a clear replica of the original

impression. The original fingerprint was not damaged by this process.

These findings made sensational news. It was feared that astute money lenders would implant borrowers' thumbprints on fresh proforma and fill in larger amounts and/or higher rates of interest. When the case was referred to F. Brewster, the then Government Examiner of Questioned Documents, he submitted himself to a test prepared by Howrah Bar. The test consisted of 12 fingerprints — a random mix of genuine and transferred ones — on a single sheet of paper. He was asked to segregate them. In one attempt, Brewster identified the forged fingerprints. He noticed three differences: the transferred prints had diffuse lines while the originals had sharp patterns; the transferred prints were impregnated with gum; and the fibres on the part of the paper with transferred prints were disoriented.

Brewster therefore concluded that an observant fingerprint expert can easily differentiate an original mark from a transplanted one.

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Science and government share anti-terrorist goals

Sir — Your news article headlined “National academies slam Bush proposal for data security” (*Nature* **419**, 769; 2002) mischaracterizes the debate here in Washington by its very title. It also leads readers to believe, erroneously, that I said that “without written guidelines, scientists can't accept [President Bush's science adviser, John] Marburger's assurances”.

The statement “Science and Security in an Age of Terrorism”, published on 18 October by the presidents of the National Academy of Sciences, the National Academy of Engineering and the Institute of Medicine, recognizes the need “to achieve an appropriate balance between scientific openness and restrictions on public information” when strategic secrets are at stake. But it also asks our government to maintain the current clear distinctions between classified and unclassified research, and recommends against poorly defined categories of “sensitive but unclassified” information that do not provide “precise guidance on what information should be restricted from public access”.

The statement also asks the Bush administration to reaffirm a national security directive signed by President

Ronald Reagan in 1985 which held that “no restrictions may be placed upon the conduct or reporting of federally funded fundamental research that has not received national security classification”.

In the wake of 11 September 2001, all of us in science and government have been forced to soberly reassess our roles concerning research touching on possible terrorist threats. No US scientist wants to publish research in a form that could be helpful to terrorists. Similarly, no US government official should want to hinder (or worse, stop) scientific research that might lead to effective tools against terrorism.

Scientists and government are listening to each others' legitimate concerns, and the government wants to enlist scientists in its anti-terrorism policies. In January, the National Academies plan to host a town meeting in which scientists, scientific publishers, national-security experts and government officials can talk face to face.

Marburger, director of the White House Office of Science and Technology Policy, is a key partner in these discussions. He informs us of the administration's concerns as well as communicating scientists' and scientific organizations' concerns to the White House. This is why I was disturbed at your implication that I said scientists couldn't rely on Marburger's assurances. That is simply wrong. The Bush administration has not yet even formulated its ultimate policy on this issue.

At the National Academies, we have never doubted Marburger's intentions or his goodwill. We share the same goal: to harness and focus the considerable energies of the science, engineering and health communities in the complex, rapidly changing challenge of counter-terrorism.

E. William Colglazier

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Peer review to select academic job applicants

Sir — Assessing the quality of candidates for academic positions has been the subject of controversy. Problems include nepotism and inbreeding, lack of impartiality among committee members — as M. Soler noted in Correspondence (*Nature* **411**, 132; 2001) — and the increasing use of impact factors as indicators of research performance (see, as one of many examples, *Nature* **415**, 726–729; 2002).

We suggest, as an alternative, that peer review could be a fairer method of research evaluation when scientists are being

assessed for new jobs. To ensure the expertise and impartiality of peers, appointment committees should include internationally recognized scientists who are not affiliated with the institution where the position is offered.

Operational costs could be kept low by use of e-mail, the Internet, videoconferencing and so on. Such cost-effective implementation would be of particular benefit to academic institutions in developing countries, which could call on a broader choice of specialists to comprise virtual appointment committees.

Rigorous criteria, relevant to the position, could be laid down before the job is advertised. These criteria might include originality of ideas, diversity of approaches, appropriateness of methods, statistical design and analyses, and so on. Candidates could then be assessed on the kind of quantitative scale that many journals use.

To that end, a comprehensive account of the candidate's research contributions, together with relevant published papers, can be presented without the journals' or the candidate's identities being revealed, so research committees can focus on scientific quality irrespective of journals' impact factors and potential personal biases. Moreover, the identity of the committee should be made public, to safeguard the impartiality of the selection process and the confidence of candidates.

Peer review has undeniably contributed to the advancement of science by providing a reliable system of quality control validated by experts. Some of the criticisms of its use (such as plagiarism and competition) are unlikely to apply to the selection of new academic staff, as it is usually a candidate's past achievements — rather than ideas, methods or data — that are under assessment.

No system is foolproof, and other criticisms of peer-review may apply to individual assessment — for example, sexism, opposing interests and lack of support for controversial ideas. However, these can be minimized by a policy of blind review, by having candidates specify potential conflicts of interest beforehand, and by including referees with different perspectives.

In summary, we believe that electronic information technologies can enhance the quality of recruitment systems in academic institutions by enabling the widespread use of peer review. This would be an improvement on current methods such as the impact factor, which is not a reliable measure of scientific excellence.

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The future of electronic data

Will the universities' own electronic repositories affect traditional publishing?

Ann J. Wolpert

"Will you manage my digital work?" is a question that twenty-first century university librarians are increasingly being asked. The answer might once have been "Sorry...", but today both university library services and university research and educational output are in the midst of fundamental change. The current response is more likely to be: "Let's talk."

At the risk of stating the obvious, the complex system of relationships and products known as scholarly communication is under considerable pressure. New information technologies (digital formats, the Internet, laptop and desktop computing, data and image capture and manipulation) have created opportunities for communication that were unimaginable in an earlier, print-constrained era.

These information technologies hold great promise for positive change in the ways that scholars, researchers and educators conduct their work. But they have also destabilized the economics of a highly complex communication system. And they challenge some basic assumptions concerning long-held roles in the value chain of traditional scholarly output. All of the stakeholders in the system are currently living in a period of experimentation, and it is not at all clear how the system will look when the dust settles.

All change

Although there is general agreement that the system of scholarly communication must change in response to new information technologies, conversations about the form that any such change might take resemble those of the proverbial blind men describing the elephant. Journal publishers view the situation in the context of revenue and production values. Authors describe their intellectual contributions and usage expectations. Librarians articulate concerns about the scholarly record and the challenge of providing short-term and long-term support to education and research. Scientists have one set of assumptions, humanists another. And the institutions that host authors, and libraries, and sometimes publishers, are trying valiantly to get a grip on the overall costs of the system. Conflicting perceptions mean that change that seems obvious to one group of stakeholders feels counterproductive to another.

Until recently, faculty and their institutions have viewed this stalemate as an annoyance rather than a threat. Networked environments had to be built anyway. Librarians seemed able to manage rising costs through judicious cancellations and collab-



ILLUSTRATIONS BY DAVID NEWTON

orations with other institutions. Faculty fussed but generally managed to teach and conduct research much as they always had.

Two events have changed this annoyance to alarm. First, teaching moved onto the Internet, and faculty and their institutions were forced to confront in a meaningful way the operational constraints of the intellectual-property environment as it affects digitally formatted materials. These legal regimes, established primarily to protect digital entertainment products in a global, networked communications environment, map poorly, if at all, to the time-honoured ways that researchers and educators teach and advance knowledge.

Second, bundled, restrictive pricing models for licensed access to formal scholarly publications began to draw the attention of university administrations. These pricing strategies significantly reduce the flexibility of institutionally based libraries to absorb price increases, and adversely affect the ability of libraries to manage the array of inter-institutional educational and research collaborations that have become increasingly important to faculty and students.

These events stirred faculty and universities to begin a series of experiments designed to further test alternatives to traditional publishing. The experiments reflect a concern with the rising cost and declining flexibility represented by conventional publishing, and may even have been initiated for those reasons. More importantly, they illuminate the evolving nature of research and research communication. There is a growing awareness of the fact that, particularly in the high-visibility emerging disciplines, the nature of

research and research documentation is changing. Examples include the increased reliance on large data sets or image sets across a variety of disciplines, and the increased commonality of large-scale data analysis. Similarly, extensive supportive documentation is often expected, whether the work is in the sciences or the liberal arts.

Built to serve

Among the more visible experiments are projects to leverage the intellectual output of faculty and institutions through the use of e-print servers and institutional repositories. E-prints are generally understood to be author or institutional archives of electronic versions of scholarly or literary works made accessible through the global Internet. E-print servers seek to address the needs of faculty who work primarily in text (working papers) and need a timely and easy way to share research and ideas across a community of interest.

It is far too early to know whether or how the self-archiving e-print movement will affect the conduct of research and teaching, or of traditional publishing enterprises. E-print servers have so far taken root in only a few disciplines, such as economics, physics, mathematics, computer science and the cognitive sciences. Many of these e-print services, such as CogPrints at the University of Southampton, UK, have been owned and operated in the conventional cottage-industry mode of traditional higher education. In general, these e-print services lack both scalability and a commitment to long-term availability.

A necessary precursor to self-archiving will be a computing environment that is far

more intuitive for the average faculty member than is currently the case. And faculty are not normally eager to function as system administrators. Worth noting is the recent relocation of the seminal 'arXiv.org' e-print server from the Los Alamos National Laboratory to Cornell University. This granddaddy of all archives moved with its creator, Paul Ginsparg, and is now maintained on a server at the Cornell University Library.

Of potentially greater long-term impact to scholarly communication is the body of non-text research and scholarly output that is growing rapidly and inexorably on university campuses. The typical doctoral-level research university currently supports considerable institutional computing capabilities, and its faculty produce and manage data, images, video, sounds, simulations, animations and a host of other media and genres. As the computing costs and support requirements of these resources proliferate in academic departments, academic institutions will be increasingly motivated to find appropriate economies of scale.

Digital data

This makes institutional repositories a more likely long-term outcome than the individually sponsored e-print server. Indeed, the current proliferation of institutionally sponsored digital repository services suggests that a variety of experiments are already well underway. The California Digital Library is developing a suite of services that should empower the entire University of California system by providing tools to house and distribute the intellectual output of university faculty.

The DSpace initiative at the Massachusetts Institute of Technology Libraries (an open-source initiative that will be implemented in a federation of between five and seven North American university libraries within a year) is designed to host and preserve a variety of text and non-text formats (see *Nature* **419**, 869; 2002). In Britain the Cambridge University Library is particularly interested in developing the capability for managing digital teaching objects on behalf of faculty and the university. And the digital collections at the California Institute of Technology (Caltech Digital Collections), Indiana University (Digital Library of the Commons) and the University of Michigan (OALster) are yet more examples of content-management services designed to support the work of faculty.

Protocols and standards for these repositories are being established through the efforts of such organizations as the Scholarly Publishing and Academic Resources Coalition, the Open Archives Initiative, the World Wide Web Consortium, and Creative Commons. Funding sources thus far include national governments, foundations and individual institutions. Almost universally, institutions are turning to their research libraries



for assistance in designing and implementing these new information services, as the work of the Digital Library Federation and the Coalition for Networked Information will attest. After all, academic research libraries have a long tradition of leveraging institutional investments, supporting the faculty enterprise, and developing sustainable preservation strategies for a variety of content formats.

A companion set of services is emerging as options for the creation of publications from large institutional repositories. Berkeley Electronic Press, for example, offers the University of California and other institutions the possibility of producing formal peer-reviewed publications through their university presses, as well as the alternative of simpler online publication strategies for those who do not need or want peer-review capabilities.

A slightly different model is Project Euclid at Cornell University. Euclid is a set of tools designed to advance scholarly communication by addressing the unique needs of low-cost independent and society journals. An alternative to traditional publishing, Euclid provides robust functionality through full-text searching, reference linking, interoperability through the Open Archives Initiative, and long-term retention of data.

Peering into the future

For the foreseeable future, it is likely that traditional peer-reviewed journals will persist in their historical niche of documenting the record of advances in disciplines. Well-regarded and heavily-used e-print services in a variety of subject areas have yet to eliminate peer-reviewed journals as the tool of choice for the permanent record of a discipline. Likewise, faculty will not lightly abandon an evaluation system that has served them reasonably well for centuries. The importance of peer recognition cannot be overstated, whether it be in the form of acceptance for publication or in the honour associated with participation in the editorial process. As peer-

reviewed journals themselves move online, however, and their print volumes disappear from library shelves under preferred licensing arrangements, it will be interesting to see whether their current market distinction becomes less obvious and less relevant.

The most interesting aspect of institutional repositories will be in the alternative forms of communication that emerge as faculty begin to exploit the capabilities of an open, interoperable system. Disciplines will have the unprecedented luxury of designing new scholarly communication systems that reflect the ways they need and want to teach and conduct research. As the volume of material in institutional repositories grows, and harvesting protocols enable the revelation and repackaging of works in new ways, the ideas of the next generation of faculty will no longer be bound by the constraints of traditional print. They may even reinvent the mechanics of peer review to take advantage of repository tools and functionality.

Meanwhile, this is a time of experimentation, and no one can be confident that their predictions will hold true. Only two outcomes look certain: first, that the status quo will not remain unchanged; and second, that research institutions have some fairly compelling reasons to encourage the availability of scholarly information and documentation on the Internet. The experiments currently underway will help to determine whether there are equally compelling reasons to provide those online information resources with the benefits of management within persistent institutional repositories. ■

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The Case for Institutional Repositories

♦ www.arl.org/sparc/IR/ir.html

DSpace

♦ <http://dspace.org/index.html>

EPrints

♦ www.eprints.org

The Berkeley Electronic Press

♦ www.bepress.com

University of Michigan OALster

♦ <http://oaister.umd.umich.edu>

MIT's ArchNet

♦ <http://archnet.org/lobby.tcl>

California Digital Library

♦ <http://escholarship.cdlib.org>

Open Archives Initiative

♦ www.openarchives.org

Research Papers in Economics (RePEc)

♦ <http://econpapers.hhs.se>

ArXiv.org e-Print archive

♦ <http://arXiv.org>

Caltech Collection of Open Digital Archives (CODA)

♦ <http://library.caltech.edu/digital/default.htm>

Indiana University Digital Library of the Commons

♦ <http://dlc.dlib.indiana.edu>

University of Southampton CogPrints

♦ <http://cogprints.ecs.soton.ac.uk>

Access to scientific literature

The web can complement libraries, but not replace them.

Wil Weston

Not everything published is on the web, despite its description as “a gigantic digital library, a searchable 15 billion word encyclopedia”¹. Only about 8% of all journals are online and only a fraction of books are available². Thus, the web is a useful research tool but it is no substitute for a library. Library services have been greatly improved by computer automation and use of the web, but the web cannot replace all of the services offered by a good library.

It is true that scientists have increased access to a growing amount of information on the web that would otherwise have required frequent trips to the library. But it is a cause for concern that “researchers are increasingly becoming biased against locating some of the higher quality research”³ simply because the desired article cannot be found on the web without payment. Apparently, a few researchers have experienced “interlibrary loan delays”⁴ and were troubled by “substantial efforts in locating the source”⁴. However, access to the web and Internet in the past five years has greatly increased the speed of interlibrary loans and document delivery. Some articles arriving through document-delivery services such as Canada’s Institute for Scientific and Technical Information (CISTI) deliver in less than 24 hours, or even within an hour. New software such as ILLiad allows interlibrary loan departments to begin making requested articles electronically available for those who prefer to remain in their office.

Restricted view

Rather than seeing the web as a replacement for their services, many academic and public libraries have embraced the web and its possibilities to enhance their services and improve resource sharing. The web is a powerful tool; librarians use it and recommend aspects of it daily to patrons who phone, e-mail or visit the library. Few libraries do not have a web presence and at least an online catalogue that can be accessed remotely. But the web is merely one of many ways of accessing information, all of which should be consulted when thoroughly researching a subject.

Frankly, anyone who relies on the web as their only research tool is not doing research of any real quality. This practice is analogous to trusting a mechanic who assures you that he can fix your car with just a screwdriver, no matter what the problem. A mechanic, like a good researcher, avails himself of all the tools needed to do his job properly.

There are several glaring problems with the notion of the web as a “gigantic digital

library”. First, the web is an uncatalogued mess and, despite what any search engine claims to do, none of them searches the entire web. Imagine a librarian telling you that there are 60 books on your subject, but he is only going to let you look at 12 of them².

Second, not all search engines or robots are created equal. Each search engine and robot searches the web differently, and some prioritize their search results according to who paid to appear near the top of the list or by the number of ‘hits’ — the popularity of the site. Imagine a librarian saying “because you used this particular catalogue to look for them we aren’t going to rank them by relevance using your search terms. You see, this publisher paid us to have their book show up higher in the list and we used their possibly inaccurate descriptors to catalogue it. Perhaps the book is on your subject, but I can’t be sure.” Librarians use not only human understanding, but also unbiased tools to answer questions.

Mixed results

Alexander Lebedev, an associate professor at Moscow State University, did an informal study called ‘Best search engines for finding scientific information on the web’⁵ in 1996, which described the functionality of selected search engines using the number of documents returned as the primary parameter for a good search engine. Most librarians would suggest that web search engines are like library resources and should be matched to the type of query being made, rather than thought of as the same type of database. Nevertheless, Lebedev’s study does point out marked differences in the number and type of documents that are retrieved by different search engines. Lebedev states: “My estimates show that the

maximum number of documents which can be found in the net is less than 10% of the number that can be (found) using a good scientific database like INSPEC or CAS”⁵.

Third, libraries have guidelines and standards for collecting all materials, as well as cataloguing them; the web does not. Although it is true that obtaining conference papers, journal preprints and technical reports is not the primary focus of any library, such materials form a valuable part of any library’s collection, together with books and journals published before the advent of the web. A researcher might not have instant access to this material, but he should be able to obtain it promptly through the interlibrary loan system. There is no guarantee that the web can do the same, or that time spent searching it will result in anything more than eyestrain.

Some indexes and databases on the web provide free access to text or at least allow one to search their holdings, such as S. Lawrence and C. Giles’ ResearchIndex. I have often referred electrical engineers and computer scientists to ResearchIndex, but always in concert with other library resources. These free online databases are a tremendous asset to researchers and are excellent tools for locating new and supplementary material. However, they are no replacement for the resources of a library, nor do they come close to mimicking the skills of a professional librarian.

Finally, the idea that the web can be a replacement for a library ignores the most important characteristics of a library. A library is not merely a collection of books, or some vast warehouse of words, books, and journals; it is part of our cultural, historical and scientific memory. With the advent of the web, libraries are now connecting and sharing their collections and resources with each other. Thus an individual academic or public library can be the access point for people to explore their world and their history, and to enlist the aid of information professionals to help guide them through their journey. ■

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♦ www.nrc.ca/cisti
♦ www.oclc.org/illiad
♦ <http://citeseer.nj.nec.com/cs>



That shrinking feeling

Lab on a Chip: Miniaturisation for Chemistry and Biology

chair of editorial board Andreas Manz
Royal Society of Chemistry. 4/yr. £395, \$596 (institutional); £90, \$136 (members of the Royal Society of Chemistry)

Harold G. Craighead

In recent years, miniaturization technologies, similar to those that produced the 'chips' of electronic integrated circuits, have been adapted to create biological sensors, devices for chemical analysis, and chemical-processing systems in a 'chip-like' format. A revolution similar to that in microelectronics may yield a proliferation of miniaturized chemical sensors, medical diagnostic devices and scientific research tools for the chemistry and biology communities. Increasing interest and activity in miniaturization technologies by industry, government labs and academic research groups has stimulated the need for, and the initiation of, the journal *Lab on a Chip*.

The aim of this journal is to provide a central resource for information and research results on miniaturization technology associated with chemistry and biology. The initial issues of the journal published a mixture of review articles and original research papers on the components and science associated with miniaturized chemical systems. It addressed 'plumbing', valves, materials and the control of fluid flow in scaled-down geometries or microfluidics. There were also papers on the methods of fabricating these microfluidic systems, which is an area of continuing development.

Lab on a Chip has attracted original papers and review articles from leading research groups. The articles are timely and of good technical quality. To date, the materials, fabrication methods and chemical applications appear to dominate the journal. Researchers and those who wish to stay informed of technological developments in these rapidly evolving areas may find it to be a significant resource. The articles are technical in nature and are appropriate for those with some technological background, as well as those actively working in the field. The journal should be attractive for researchers in the areas of microfluidics, sensors and microchemical analytical systems.

The editors of other technical journals in the areas of lab automation and chemical engineering are recognizing the importance of the miniaturization of analytical systems. Traditional scientific journals will continue to attract leading papers on the scientific advances in miniaturized systems, but *Lab on a Chip* concentrates on the technological



issues associated with fluid handling and the miniaturization of chemical systems. Its 'research highlights' section, which reviews recent publications in the area from other journals such as *Nature*, *Analytical Chemistry*, *Sensors and Actuators* and the *Journal of Microelectrochemical Systems*, is a helpful central source for keeping abreast of such developments.

Timely publication is important for the use of information in areas of rapid technological development. The journal has an electronic component and promises that papers will be available on the web 4–6 weeks before print publication. It accepts submissions electronically and aims for a rapid turnaround with publication in 120 days. ■ Harold Craighead is in the School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14853, USA.

♦ www.rsc.org/is/journals/current/loc/locpub.htm

Biological molecules at large

Macromolecular Bioscience

editor Ingrid Meisel

Wiley-VCH. 12/yr. 3,564 euros, \$4,444 (as part of a journal package); 998 euros, \$1,198 (separate from 2003)

David L. Kaplan

There is increasing interest and research activity at the intersection of biological synthesis, polymer science, and materials science and engineering. This new journal, *Macromolecular Bioscience*, focuses on the interface between polymer science and the

biological sciences. It aims to include studies from physical and chemical perspectives with relevance to biotechnology, as well as topics related to medical impacts. Physicists, chemists and engineers are the primary target audience.

The journal has been published within the *Journal of Macromolecular Chemistry and Physics* as a second section at the back, but from 2003 it will appear as a separate journal. This 'piggy-backing' is a useful approach to ensure wider visibility and to promote cross-fertilization from biological perspectives between the disciplines of chemistry and physics.

The summary page for each issue includes highlights such as titles, authors, abstracts and a key figure or table from each article, which facilitates the rapid scanning of contents. The contributions to the journal in the first year and a half emphasize biopolymer synthesis, enzymatic methods in polymer formation, phase control in biopolymers or hybrid synthetic/biosynthetic polymers, and related areas. Forays into topics such as tissue engineering, diagnostics and bioinformatics have not yet been featured, although they are proposed for broadening the scope of the journal.

The initial quality of contributions has been strong, and the inclusion of reviews, full papers and communications (short papers) is a selling point. The new journal was launched in February 2001 and nine issues have been published so far, with four to ten articles per issue. It will be published monthly from 2003, and the number of papers per issue probably needs to increase if it is to make an impact.

The journal has attracted a strong international advisory board that is actively contributing reviews and new papers to the journal, and this will clearly help to ensure short-term interest. Highlights include reviews on polyhydroxyalkanoates and block co-polymers from poly(L-lactide) and polyoxyethylene leading to nanostructures, and full papers have included studies of new cellulose derivatives, control of cholesterics from biopolymers, enzymatic digestion of new co-polymers of L-lactide and cyclic carbonates, synthesis of hydroxypropylcellulose, and enzymatic degradation of poly(L-lactide).

With the recent proliferation of journals focused on the theme of biological polymers, the likely long-term impact of this entrant into the field is not clear. However, the journal is off to a solid start and fits well within the Macromolecular Science series from Wiley-VCH. It might be useful to maintain the focus as currently offered in the contributions instead of broadening into the full range of proposed topics, because this will match most closely with the macromolecular chemistry and physics audience. ■



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 ♦ www.wiley-vch.de/publish/en/journals/newJournals/2127

A home for proteomics data?

Molecular and Cellular Proteomics
 editor Ralph A. Bradshaw
American Society for Biochemistry and Molecular Biology. 12/yr. \$350, \$75 (members of the American Society for Biochemistry and Molecular Biology), online access free this year
 Matthias Mann

Suppose you have just finished an exciting study involving the identification of hundreds of proteins that are part of a complex, or that change in response to some condition. Where can you publish your results? This is a real problem. If you send them to a biological journal, the reviewers may tell you that that you should get functional data for the proteins, which could take you the rest of your career. If you send them to a technologically or analytically orientated journal, they will say that your methods have already been adequately described in the literature and so your paper is not very interesting. So you may just sit on your proteins, and have them worked up by incoming graduate students over the years, which would be a loss to the scientific community.

Enter *Molecular and Cellular Proteomics* (MCP), a spin-off from the *Journal of Biological Chemistry* (JBC), the official outlet for the American Society for Biochemistry and Molecular Biology. This new journal is dedicated to studies just like the one described above, and therefore meets a real need.

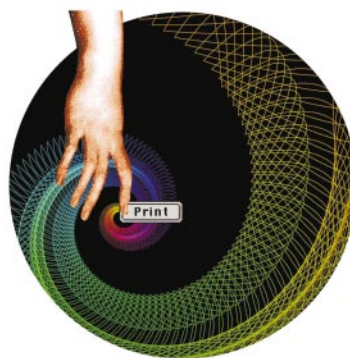
Judging from the articles published in the

first few issues, the mission statement and the composition of the editorial board, MCP takes an expansive view of proteomics, embracing subjects such as bioinformatics related to proteomics, protein databases, two-hybrid methods for protein interactions, two-dimensional gel studies, and mass-spectrometric methods.

There are three categories for original articles: research, database and technology. Another new feature is the online site, which, as well as displaying an electronic version of the printed journal, is intended to function as an associated database for the proteomics investigations described in the papers. This material can be much more extensive than usual, and MCP is working on navigation and visualization features. The quality of papers published so far is good, with a few groundbreaking papers already in the bag.

Publication speed is, in my experience, quite rapid, with the additional advantage that accepted papers are put on the web immediately, even while the paper is being edited into the final version. This can be quite useful for authors in a publishing race.

Where should MCP stake its claim in the publishing food chain? The premier position in proteomics is taken by *Nature Biotechnology*, and breakthrough biological results will also probably be reported elsewhere. MCP is well positioned to become to proteomics research what JBC is to biological research —



a standard place for solid results that have undergone stringent peer review and that will be easily accessible to almost everyone. For this to happen MCP needs to maintain or even strengthen its reviewing standards, and focus on the quality of papers rather than the number published if it is to achieve an impact factor similar to that of JBC.

Once the journal is firmly established and its identity is clear in everybody's mind, there should be no shortage of papers as proteomics methods and proteomic-scale experiments become more commonplace. The *Journal of Proteome Research*, launched at almost the same time by the American Chemical Society, is likely to concentrate more on technological advances, and *Electrophoresis* and *Proteomics* will probably continue to be more focused on the two-dimensional-gel community.

The format of MCP articles varies somewhat, and standardization would make the journal more visually appealing. In extension of its online features mentioned above, MCP could perform a great service by helping to establish some standard as to how proteomics data collections are published and visualized so that they can actually be used by biologists.

In conclusion, faced with the dilemma I outlined at the start, I would encourage you to publish your proteins in MCP. Scientists with similar data should think 'out of the box' and submit their proteomics data for the community to use, and now there is a place for them to do it. ■

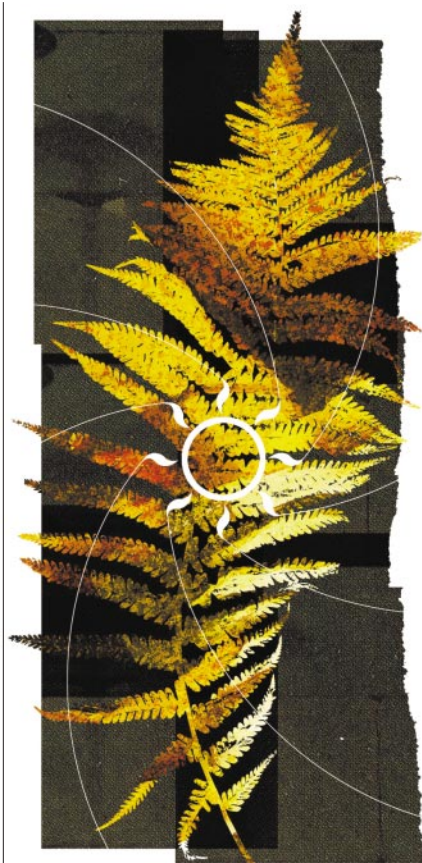
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♦ www.mcponline.org

Shining a light on molecules

Photochemistry and Photobiological Sciences
 editors-in-chief Frank Wilkinson & Tamás Vidóczy
Royal Society of Chemistry. 12/yr. £723, \$1,091
 Robin M. Hochstrasser

The introduction of a new journal often signals the emergence of a sub-field or scientific direction that needs to establish a voice of its own, a place where specialists can air their views. A new journal can provide a balance when trends narrow the impact of journals that were intended to have a broader context. Specialist journals can also be healthily unimpressed with hyperbole, and tell it like it is. *Photochemistry and Photobiological Sciences* aims to do just this in fields related to "any



aspect of the interaction of light with molecules, supramolecular systems or biological matter”.

Experimental and theoretical principles governing the interactions of photons with molecules, and the resulting behaviour of electronically excited states, are readily transferable between applications in chemistry, materials science, biology, atmospheric science and medicine. Although the properties of excited states are profoundly influenced by chemical variations and the surrounding medium of the molecule, there are systematic ways to understand these variations, regardless of the complexity. This gives the journal a meaningful focus and a good chance of contributing to the more effective flow of knowledge and expertise between these fields.

Photochemistry and Photobiological Sciences does not aim to compete with the highest-impact journals, but rather to provide a medium for researchers to disseminate solid contributions on photoscience that form the framework of excited-state chemistry. The journal has a chemical flavour and may be read by organic photochemists as frequently as by photobiologists. I can certainly envisage browsing it to discover interesting optical properties of molecules and biological systems, and I would expect others whose work touches on molecular excited states to find something of interest in it.

Although published by the Royal Society of Chemistry, it has an international appeal and has become the official journal of the European Photochemical Association and the European Society for Photobiology — members of both can make large savings on the subscription price. The editors-in-chief are from Britain and Hungary, and the associate editors are based in a range of European countries as well as Canada and the United States.

Besides conventional papers, the intention is to publish short communications on either fascinating photoscience or technical advances, along with review-style perspectives, which will be commissioned by the editors. The electronic resources of the Royal Society of Chemistry provide for electronic submissions, easy access to the journals and free electronic reprints. The editors promise an average of 95 days between submission and publication. Although I expect that this will depend on how long it takes referees to respond and how many referees are used (at least two, I hope), the commitment to reduced publication times is excellent news. The free use of colour will also be attractive to prospective authors.

In summary, the journal spans a range of fields but has a sharp focus on photo-related phenomena. Nevertheless, if high standards of refereeing and editorial judgement can be established, it should be of interest to anyone involved in photochemistry in its widest sense and become a useful specialist journal that fills a gap. ■

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♦ www.rsc.org/pps

♦ www.unibas.ch/epa

♦ www.pol-us.net/ESP_Home/abt_esp.html

Adapting to a diverse world

Organisms, Diversity and Evolution

editors-in-chief Gerhard Haszprunar, J. Wolfgang Wägele & Joachim W. Kadereit
Urban & Fischer. 4/yr. £182, \$302, 255 euros (institutional); £70, \$122, 152.40 euros (individual)

Andy Purvis

The title of this new journal certainly leaves plenty of scope, and promises a diverse set of papers. The taxonomic breadth is indeed commendable: the issues I saw featured bacteria, yeast, plants and six metazoan phyla. There is also a range of article types: reviews, original papers and purely taxonomic papers, with the last of these being published online and abstracted in the printed version

— an excellent compromise between authors' and publishers' views about how long papers should be. Papers show a pragmatic mixture of methodologies, too, with no sign of editorial dogma; for example, phylogenies are estimated from molecular and morphological data in about equal numbers, and using parsimony, likelihood and distance approaches.

Not all of the promised diversity has materialized so far, however. Straight-forward systematics and phylogenetics predominate, with some comparative anatomy, character-evolution studies and distributional work, but there is no science policy news or palaeontology, and surprisingly little by way of formal hypothesis testing. Some papers were impressive and thought-provoking, but I was disappointed by the level of analysis in others, and there was little methodological innovation. Such patchiness is to an extent inevitable — almost every new journal's call for papers is met by an emptying of desk drawers — and will hopefully peter out as the journal receives more submissions.

Having said that, *Organisms, Diversity and Evolution* has a particularly narrow author base. It is the journal of the Gesellschaft für Biologische Systematik, after all, so it is hardly fair to criticize it for not being fully international. But all three of the editors-in-chief are based in Germany



(and one of them is an author on four of the 21 papers I saw); half of the first authors hail from Germany, too, and most of the rest are from Western Europe. If the journal wishes to have an international impact, it will surely need to broaden its author base.

There are certainly good reasons why authors might want to consider this journal. There are no page charges, for a start. Despite this, production values are pretty high: the layout is easy on the eye, and the figures are crisp and clear (although colour figures are only published electronically). Authors also receive 25 free offprints. Publication time seems fairly fast — about five months from submission on average — although this will presumably rise as resubmissions work their way through the system. I can't see many individuals wanting to buy the journal, however: it covers too wide a range of taxa (so far) and too narrow a range of questions, and is anyway priced more for institutions.

If it can keep its publication time down, keep its production values high and broaden its geographic pull, then *Organisms, Diversity and Evolution* has every chance of establishing itself as a valued library journal. It will be interesting to see whether it begins to narrow its focus, as many previous journals have, or maintains its diversity of subject matter and approach. ■

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► www.urbanfischer.de/journals/ode/organism.htm

In the land of the giants

Comparative and Functional Genomics

editor-in-chief Steve Oliver
Wiley. 6/yr. \$240 (institutional);
\$180 (individual)

Greg Elgar

Less than a decade ago, some expression data, a few kilobases of sequence and an alignment were still sufficient to wring a publication out of *Science*, *Nature* and other high-brow publications. Nowadays, of course, scientists need to come up with whole genomes. Such is the pace of progress that even publication in more specialized journals such as *Genomics* and *Genome Research* requires mega- or even tera-buckets of data, beyond the scope of the average lab. For those researchers (most of us actually) who work in the smaller lab environment, it is vital that public databases are mined and interpreted to their full extent, both to enrich our knowledge of specific genes or pathways and to allow the design of more effective experiments. It is equally import-

ant to be able to shout about it and let others know just how fascinating and groundbreaking your research really is.

The use of comparative genomics as a means to investigate gene function is a direct result of the wealth of genomic data in public databases, and has led to its widespread application in dissecting gene function. It is therefore essential that this new discipline has a dedicated journal in which to disseminate these findings. *Comparative and Functional Genomics* fills the void and is appropriately forward thinking and looking in concept and design. As well as presenting a (currently slightly thin) selection of original articles and reviews, it includes comprehensive coverage of meetings in the field, as well as a particularly useful 'Current awareness' section, which lists recently published reviews and papers by subject matter.

Despite the organization and clarity of many large public databases, a huge amount of data and tools lurk in an enormous number of 'genomics' websites — well over half-a-million pages according to a search on Google (<http://www.google.com>). Perhaps in recognition of this Internet genomics jungle (or perhaps jumble is more appropriate), *Comparative and Functional Genomics* regularly carries a website review, which usually involves a jolly good trawl through some of the myriad sites surrounding a particular topic, such as cancer.

The quality of the reviews and original research published in the journal is high,

and no doubt once it starts popping up in the citation indices, the quantity of submissions will also increase. This is assured, as many researchers are starting to combine *in silico* comparative analyses with functional annotation, a necessary and important consequence of genomic sequencing. There is clearly a demand for this type of journal, and until recently the options for publishing in this area have been very limited. I only hope that *Comparative and Functional Genomics* doesn't outgrow the needs of the scientist who is content to work on genes, rather than on whole genomes. ■

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► www.wiley.co.uk/wileychi/genomics/cfg.html

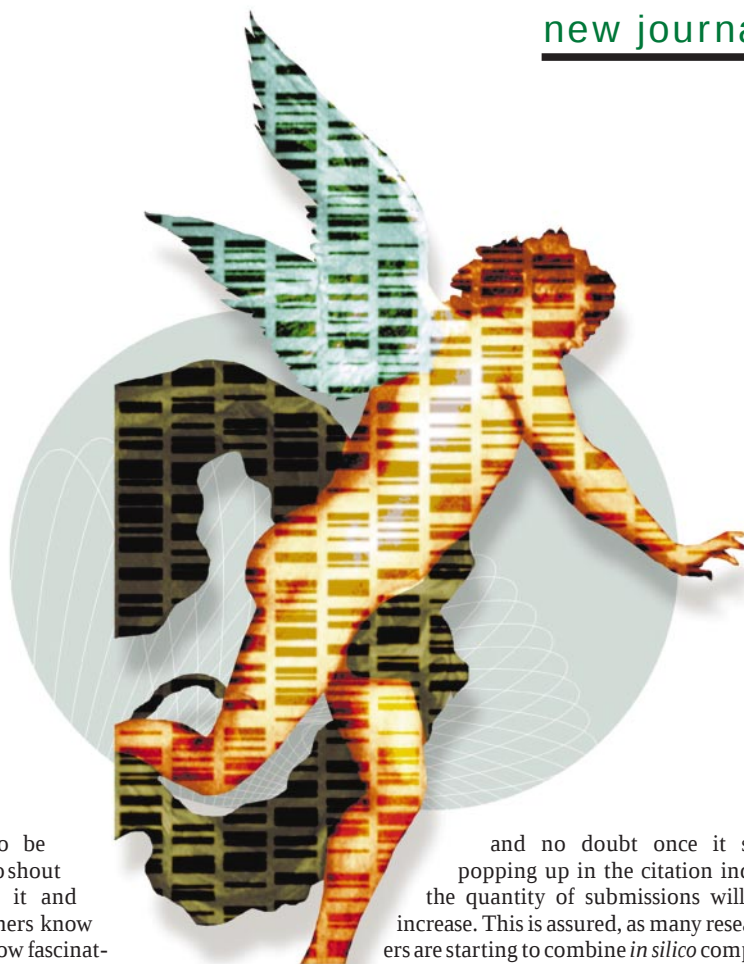
Signs of life in planetary science

Astrobiology

editor Sherry L. Cady
Mary Ann Liebert Inc. 4/yr. \$286
(institutional), \$219 (institutional online only); \$183 (individual), \$138 (individual online only)

Monica M. Grady

Astrobiology (sometimes called exobiology or bioastronomy) is the grand-sounding study of the origin, evolution, adaptation and distribution of past and present life in the



Universe. It is an interdisciplinary subject that has only fairly recently attained scientific respectability. Gathered under the banner of astrobiology is a rich assortment of scientists, including astronomers, biologists, chemists, geologists, microbiologists, palaeontologists and planetary scientists, and astrobiology provides a place for them to exchange ideas and information. The recognition of this discipline has probably changed neither the subject matter of the research carried out, nor altered the way in which the research is done, but perhaps the label of 'astrobiologist' has helped to shift the emphasis of particular projects.

The diverse subject matter of astrobiology has resulted in the publication of papers across a variety of journals, and — efficient online search engines notwithstanding — unfamiliarity with a subject could result in contributions being overlooked. As a corollary, papers that have a cross-disciplinary focus might not find a comfortable home in journals that are becoming increasingly specialist. It is therefore extremely timely that the discipline should spawn its own journals.

Astrobiology is aimed at professional scientists but, because of the nature of the subject, the papers are more general than are usually found in the scientific literature. This does not detract from the journal — indeed, it could be an extremely valuable aspect that is worth developing by the publication of review papers aimed at informing the community.

The journal was launched in spring 2001, and is beautifully produced, with an eye-catching cover. It seems to have successfully attracted both the microbial- and planetary-science communities, with papers covering, among others, the silicification of bacteria, the chemistry of adenine, the evolution of photosynthetic organisms on Earth, and the exploration of Mars and Europa. Eventually, it is to be anticipated,

there will be contributions with a more astronomical bias.

The journal offers 'Astrobiology Literaturewatch', a documentation of papers published on astrobiology. The list, which runs to 26 pages in the winter 2001 printed issue of the journal, is certainly thorough, and provides a useful summary of the current state of the literature, but it is much more useful as an online resource. As is now expected, *Astrobiology* is also available electronically.

In this rapidly growing and broad field, it is entirely appropriate that a new journal should emerge. It will be interesting to see how, like life itself, it evolves, adapts and survives.

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♦ www.liebertpub.com/ast

Noise worth listening to

Fluctuation and Noise Letters

editor-in-chief L. B. Kish

World Scientific, 4/yr. \$280 (institutional);

\$112 (individuals, print only)

Katja Lindenberg

A surge of interest in systems on the small scale is to be found everywhere in the scientific and popular literature. This interest arises from the context: many of us find biological systems and devices on the nanoscale to be intriguing or fascinating. The enormous progress in the understanding of the nanoscale world has been fuelled by developments in fields such as nanotech-



nology, protein folding, molecular motors, colloidal chemistry and self-assembly, to name but a few. In turn, the rapid strides in these fields are made possible by the vastly improved ability to study and manipulate systems on such a small scale, by the ever-growing computational power to simulate their behaviour accurately, and by the parallel development of new theoretical tools.

Of central interest in much of this research is the observation that on this small scale the effects of fluctuations become significant and even crucial. And, far from being a disturbing side-effect, it is now clear that noise may have an important or even an essential role in the operation of small-scale devices, leading to organized behaviour that would not occur in the absence of noise. One example is the world of molecular motors, in which the energy in random fluctuations is harnessed to produce directional motion of proteins along microtubules.

It is not surprising then that a search on the keywords 'noise' and 'fluctuations' yields tens of thousands of research articles each year in the physical, chemical and biological literature. Even if the search is narrowed to the half-dozen most widely read physical journals (such as *Physical Review*, *Physica* and *Europhysics Letters*), there are still hundreds if not thousands, and a considerable subset of the papers deals with the constructive role of noise. Most of those involved in research on noise and fluctuations in this context have had to search much more narrowly, usually by the names of authors we are familiar with, to keep up with this literature. This is not a particularly



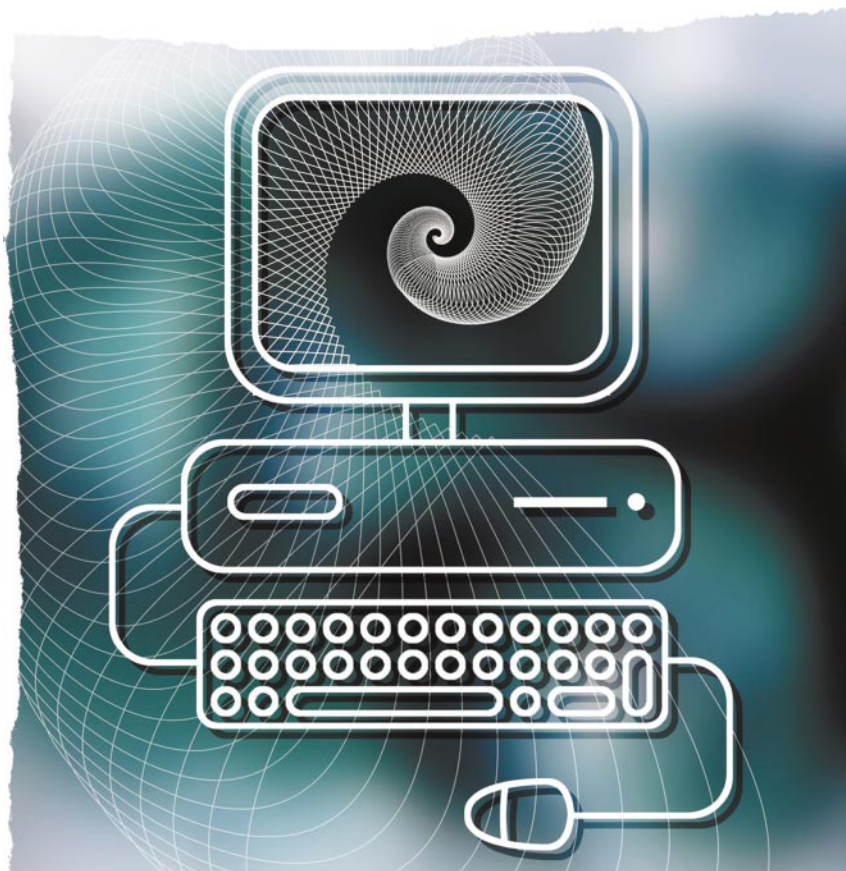
effective method, and it certainly doesn't encourage new players into the field.

The journal *Fluctuation and Noise Letters* provides a forum for concentrated publication of this literature. Before its appearance on the scene there was not one journal in which the community of 'noise scientists' (in the sense described above) could converge; it fills an important and previously unoccupied niche. The editors are an outstanding multidisciplinary and multicontinental group who have a serious scientific interest in working towards the journal's success.

Fluctuation and Noise Letters is only a little over a year old, and yet has already attracted many publications from a distinguished set of authors. Practically every paper so far has interested me. It offers an appealing variety of papers (letters, current opinions and reviews), is highly effective, efficient and selective in its peer-review process, and is attractive in format, including the easy inclusion of colour figures. Although paper copies are available, the electronic accessibility is most attractive. Now those of us who find this an extremely useful and interesting venue must encourage our libraries to subscribe. ■

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♦ www.worldscinet.com/fnl/fnl.shtml



..... Growing up with quantum computing

Quantum Information and Computation

Coordinating managing editor Hoi Kwong Lo

Rinton Press. 7/yr. \$260 (institutional);
\$140 (individual)

Isaac Chuang

Physics and computer science have given birth to a new field: quantum computation and quantum information. And just as proud parents with newborn children may find *Parenting* magazine appearing in their mailboxes, researchers in this field now have *Quantum Information and Computation*, a timely new journal from Rinton Press.

This journal, just over one year old, serves a nascent community that was originally founded in the mid-1980s on the principle that information and physics are fundamentally intertwined at their deepest levels. The spark that ignited worldwide interest in this insight sprang forth in 1994 with Peter Shor's discovery of a theoretical way to use quantum-mechanical resources to unravel a mathematical problem at the heart of electronic commerce and cryptography. The

heyday of quantum computing followed, with rapid invention of ways to combine the results of classic information theory such as error correction with quantum physics. Experiments also successfully realized quantum algorithms and protocols including quantum state teleportation, using techniques from optics, nuclear and atomic physics, and even exotic chemistry. Eight years on, as maturity is beginning to descend, the excitement is still palpable and the young field is starting to walk — but where will it learn to talk?

The appearance of *Quantum Information and Computation* fills this need in the community, establishing a middle ground between computer science, physics and the publication forums of other disciplines. Quantum-computing papers currently clamour for time and attention all the way from *Science* and *Nature* to the proceedings of the IEEE *Foundations of Computer Science* and the ACM *Symposium on Theory of Computing*. Also, the field has gradually developed its own language and style, challenging the limitations of venues intended for general audiences. In *Quantum Information and Computation*, papers have a natural home where authors will finally be free to assume a basic knowledge of concepts, such as quantum circuits and computational complexity.

Readers will also be pleased to find the

journal well balanced and populated with high-quality articles of generous length. The members of the editorial board are accomplished and respected leaders in the field, representing both theoretical and experimental work, and including experts in both physics and computer science. This careful balance is reflected in the contents: the first issue is a beautiful four-part treatise on entanglement in all its theoretical aspects (providing a much-needed entrée into the subject), and another early issue elegantly assembles 14 expert articles covering all the main implementation schemes for quantum computers, including an inspired piece by David DiVincenzo on 'Dogma and heresy in quantum computing'. The journal publishes tutorials as well as regular articles, and features in-depth reviews of books in the field, plus a regular and lively 'webcorner' that lists online links to active research groups, upcoming conferences and workshops.

Judging from the first ten issues, *Quantum Information and Computation* is here to stay, and will find a warm welcome in the quantum-computation and quantum-information community. ■

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♦ www.rintonpress.com/journals/qic

From bench to bedside and back

Cell Cycle

editor-in-chief Mikhail V. Blagosklonny
Landes Bioscience. 6/yr. \$80 (individual),
\$250 (institutional); free in 2002

Cancer Biology and Therapy

editor-in-chief Wafik S. El-Deiry
Landes Bioscience. 6/yr. \$80 (individual),
\$250 (institutional); free in 2002

Michelle D. Garrett

Finally! A journal dedicated solely to my favourite topic — the cell-division cycle — and what a discerning editorial board to boot. For someone working in this field, *Cell Cycle* was a joy to read, and for newcomers it will be invaluable in getting them up to speed on an ever-expanding area of research. But is it really necessary to have a whole journal dedicated to one particular cellular activity?

Actually the cell cycle is such a key cellular process that I am surprised that such a journal has not been developed before now. Besides, the cell cycle has clear applications to many other areas of basic biological research, including development, differentiation, cell death, senescence and disease. Cancer in particular often involves genetic alteration of one or more of the cell-cycle regulators. *Cell Cycle* therefore provides a forum for researchers in these areas to present new findings that involve the cell cycle, so it is a truly interdisciplinary journal of interest to a large readership.

The format of *Cell Cycle* is simple: there is an 'Editor's Corner', 'Views and Commentaries', 'Reviews' with a focus and finally a section presenting 'Experimental Papers'. The Editor's Corner provides a space where current issues can be highlighted, perhaps to do with a particular scientific topic, such as the interaction between basic cell-cycle research and cancer biology. It is also used to profile individuals, both past and present, who have made key contributions to research on the cell cycle and related areas. This is a valuable use of editorial space, as it reminds us of the history of this ever-growing field and of the individuals who have played a part in it.

Views and Commentaries contains reviews and comments on selected papers from the Experimental Papers section. This is a particularly useful exercise because these short articles provide background information that may not have been in the experimental paper. They are written by experts in the field who provide insight on the paper they are reviewing. Like many other journals, *Cell Cycle* publishes conceptual review articles. The added benefit here is that often all the reviews in an issue cover different aspects of one particular area of research in

the cell-cycle field, such as mitosis, proteolysis and the cell cycle, giving the reader an overall view of the area.

The presentation of Experimental Papers has the added advantage of providing a forum where new methods can be reported. This is particularly relevant to cell-cycle research, where advances in the use of experimental techniques such as flow cytometry have been essential for the progression of the field.

As mentioned earlier, cancer is a disease in which the cell cycle is frequently misregulated. *Cancer Biology and Therapy* provides a comprehensive look at this and other molecular and cellular aspects of cancer biology, along with current and future cancer treatments. The readership for this journal will be broad, encompassing all who wish to learn more about cancer and current thinking on both its diagnosis and treatment. This journal will be of interest to the clinician on the front line of cancer treatment and also to the bench scientist at the forefront of biological research into this disease — it brings together basic cancer biology and the clinical aspects of this disease.

The layout of the journal follows the generic pattern of editorial comments, major reviews, original research papers and book reviews, but has several delightful nuances. The first of these, the 'Bedside to Bench' report, in many ways epitomizes the journal, as it has a very translational feel, highlighting a specific clinical area and then discussing the basic aspects of molecular and cellular biology that it may involve. Each of these articles opens with case reports on particular patients, an excellent reminder to all of us of why we are involved in cancer research and treatment in the first place. The Bedside to Bench concept is, in many ways, the opposite of other journals, which often

provide reports that go from the bench to the bedside, and I found it refreshing.

The original research papers presented in this journal are at the forefront of both basic biological research and the treatment of cancer. The added nuance here is that many are accompanied by a commentary that provides both a critique of the research paper and valuable background information that the reader may not be aware of. The commentary acts as a 'journal club' on the paper — very useful indeed, especially if you are a newcomer to that particular area.

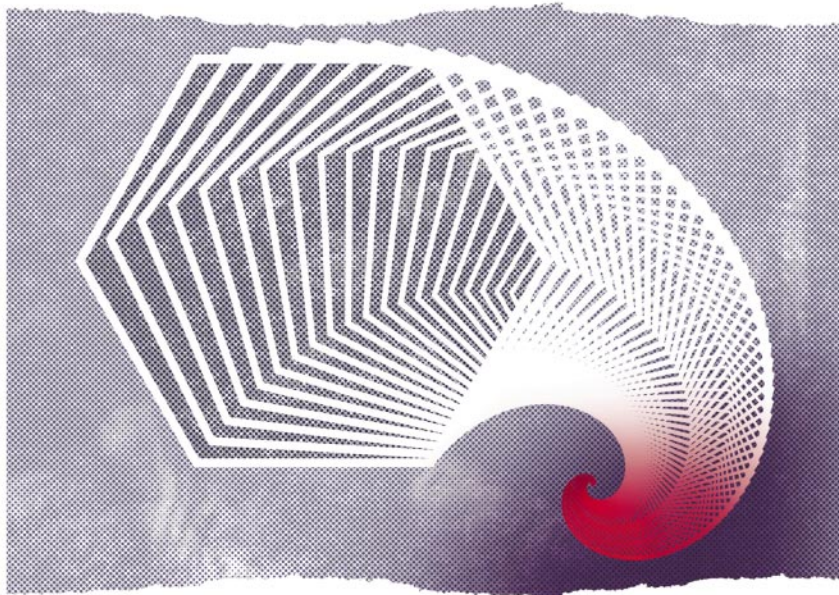
Another unique feature is the 'Research Philosophy' section, which covers ethics and thinking on science, along with its practice. This is a topic that deserves its own section and which in other journals is often absent or relegated to occasional discussion. *Cancer Biology and Therapy* deserves a round of applause for highlighting an extremely important aspect of scientific research that in my opinion should be read by both newcomers to the field and the old hands.

To conclude, *Cancer Biology and Therapy* provides an excellent forum for the presentation of new information on the basic biology, diagnosis and treatment of cancer, the second most common cause of death worldwide. *Cell Cycle*, on the other hand, is a more specialist journal, but should be essential reading for anyone who has even a remote interest in the cell cycle, from undergraduate student to head of department. Both of these journals are currently only available online, and access is free at the moment.

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♦ www.landesbioscience.com/journals/cellcycle.html

♦ www.landesbioscience.com/journals/cancer.html



The radical life-giver

Doris Abele

The introduction of oxygen into the Earth's atmosphere was a double-edged sword. It provided a fuel that would allow the evolution of complex organisms with high energy demands, but also represented a new source of toxins. Oxygen-respiring eukaryotes needed not only to develop machinery to harness the power of oxygen, which they gained through the acquisition of mitochondria, but also to build up defences against its toxic effects.

Oxygen has been a trouble-maker from the very beginning. Although by nature a sluggish reactant, it has a tendency to 'radicalize', forming incompletely reduced reactive oxygen species (ROS, such as $O^{\cdot -}$, H_2O_2 and $HO^{\cdot}$), which are highly potent oxidants. Within the cell, ROS can cause genetic degeneration and physiological dysfunction, eventually leading to cell death and progressive ageing of the organism.

Mitochondria evolved as the specialist power plants of eukaryotic cells, and mitochondrial respiration is one of the most amazing examples of the economizing principles of evolution. But a primary function of mitochondria may have been to compartmentalize respiration, to protect the cytosol from the damaging side-effects of oxygen metabolism. ROS formation — which occurred even at the low oxygen levels that prevailed a billion years ago when the earliest multicellular animals appeared — could have posed a serious problem. However, in these diffusion-limited species with only rudimentary circulatory systems, mitochondrial respiration apparently kept cellular oxygen concentrations high enough to cover tissues' oxygen demands, but low enough to minimize ROS formation. Only as oxygen levels rose, and more complex animals evolved, were more stringent antioxidant defence mechanisms required.

In air breathers, oxygen uptake is predominantly controlled by monitoring CO_2 concentrations in the blood. This makes sense, as the

oxygen concentration in air is virtually constant. For water breathers, however, oxygen levels are the prime factor that controls ventilation rates, and thus the availability of oxygen to tissues and cells. As oxygen's concentration in water is typically 30 times less than in air, its tension decreases drastically when even small amounts of the gas are consumed, whereas CO_2 tension in water is stabilized by bicarbonate buffering. Many marine animals keep respiration and metabolic performance constant against fluctuating environmental oxygen levels (oxyregulation), but in phylogenetically older species the rate of oxygen uptake mirrors the ups and downs of oxygen in their environment (oxyconformity). Although the latter may sacrifice scope of activity and performance and live at a slower pace, oxyconformity is obviously advantageous when it comes to surviving environmental hypoxia.

By initiating a metabolic slowdown, oxyconforming marine invertebrates can survive extended periods of severe oxygen deficiency in a metabolically dormant state. For reasons unknown, the ocean quahog (*Arctica islandica*) vacates the oxygenated bottom water and burrows into anoxic sediments. The buried clams reduce their heart rate to as little as 10% of routine activity, and energy demand is lowered accordingly. By making use of this quiescent state, the quahog can reach the grand age of 220 years. Upon surfacing, the quahog's mitochondria increase the respiration rate, leading to an increase in ROS formation and presumably inducing antioxidant enzymes. Their voluntary anoxic mudbaths might be a trick to beef up their stress defences.

Oxyregulators liberated themselves from the energetic limitations imposed by oxyconformity and flourished in energy- and oxygen-rich environments. But this is a one-way road. In vertebrates, paradoxically, hypoxia elicits massive ROS formation as a cellular stress response. Formation of ROS under local hypoxia is known from a wide range of human pathologies, such as ischaemic brain damage in stroke patients. In higher animals (fish upwards), hypoxic mitochondrial ROS production functions as an alarm signal and induces reactions to conserve the cellular energy balance. In mammals, 'hypoxic' genes are transcribed in response to oxygen stress, expressing factors that switch on anaerobic energy production and induce vasodilation to get blood into hypoxic areas. Could this be the modern equivalent of climbing into the anoxic mud to stock up on stress defences?

When exposed to critical warming, cold-blooded marine animals experience functional hypoxia in oxygen-saturated water. Warming a cold-blooded animal increases its respiration rate and thus mitochondrial ROS production,

Toxic oxygen

A primary function of mitochondria may have been to compartmentalize respiration, thus protecting cells from the damaging side-effects of oxygen metabolism.

while at the same time the increased energy demand results in oxygen deficiency. Functional hypoxia at high temperature is risky, as it overrules at least the peripheral oxygen-sensing systems and deprives hypoxia-tolerant animals of the possibility of systemic metabolic slowdown. As the animals go beyond their heat-stress limit, hypoxia impairs mitochondrial electron transport because oxygen, the final electron acceptor, is limited. Oxidative injury ensues from auto-oxidizing components of the redox chain. To make matters worse, hypoxic acidification sustains high mitochondrial proton-motive force and further stimulates leakage of electrons from the respiratory chain. Inflowing oxygen is rapidly reduced to superoxide, damaging the mitochondria themselves and eventually causing them to initiate apoptosis — cellular suicide.

In warm-blooded animals, cooling of the brain by as little as 2 °C can reduce ischaemic brain damage after stroke. Although many mechanisms may be involved in the beneficial effect of brain cooling in stroke patients, part of it is due to reduced neuronal oxygen turnover, reflecting an enforced slowdown of neuronal metabolism. This ameliorates hypoxia-induced ROS production by ischaemic brain mitochondria, preventing further oxidative injury of damaged structures.

Thus mitochondria, which began as oxygen-quenchers in early oxyconforming organisms, have become metabolic accelerators in higher-tuned oxyregulating invertebrates and fish, which risk driving their bearers into toxic oxygen injury. In higher animals, however, mitochondrial ROS formation also participates in cellular sensing and signalling during functional hypoxia, to oppose tissue damage. But when even these defences prove inadequate, mitochondria catalyse the destruction of the cells that contain them. Living with oxygen is certainly a dangerous affair. ■

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FURTHER READING

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Burrowing under: the long-lived ocean quahog.

The wildfire factor

David Schimel and David Baker

Events such as wildfires, occurring on a tiny area of the globe, can have a huge impact on the global carbon cycle. This much is plain from investigation of the terrible fires that afflicted Indonesia five years ago.

In 1997–98, the growth rate of carbon dioxide in the atmosphere doubled, reaching the highest on record. As even casual television viewers could have guessed, a contribution to the increase might have come from the wildfires occurring in Indonesia at that time, which burned for months and were widely shown around the world. On page 61 of this issue¹, Page *et al.* go beyond guesses, and calculate that in fact much of the inferred increase in atmospheric CO₂ can be accounted for by these fires. The authors estimate the emissions to have been $0.8\text{--}2.6 \times 10^{15}$ grams — petagrams (Pg) or gigatonnes — of carbon. This is equivalent to 13–40% of the annual emissions from anthropogenic fossil-fuel combustion.

Page *et al.* used satellite imagery and field measurements for Indonesia to estimate the burned areas and carbon losses. The burned areas included vast deposits of peat, in some cases 20 m thick, which had lost between 25 and 85 cm of their depth through fire. Most of the carbon released came from this source, not from the burning of forest trees. To put the size of the emissions from these fires in another way, they were comparable to the global carbon uptake by the terrestrial biosphere in a typical year — yet they came from a relatively tiny area of the globe.

In a parallel study in *Global Biogeochemical Cycles*², Langenfelds *et al.* used atmospheric measurements to investigate the effects of biomass combustion on the global carbon cycle. They estimated the proportion of CO₂ released by fire from the ratios of hydrogen, methane and carbon monoxide. Fire emits these other gases in relatively consistent ratios, so CO₂ combustion emissions can be derived from measuring them. Langenfelds and colleagues' approach cannot identify precisely where the fires occurred, but it does implicate fire as a significant contributory factor to changes in CO₂ emissions. Their estimate of the amount released in 1997–98 (0.8–3.7 Pg C) overlaps with the figures calculated by Page *et al.*¹. Together, these two papers provide strong evidence that the Indonesian wildfires made a large contribution to the leap in atmospheric CO₂ levels in 1997–98.

Rough confirmation of both the magnitude and location of the carbon emissions comes from a different approach again — spatial analysis of global CO₂ concentrations in the atmosphere, which we have used to

compile Fig. 1. Sources and sinks of CO₂ are estimated from observed spatial patterns and temporal changes in atmospheric CO₂, using the approach known as inverse modelling³. By itself, this approach can provide only tentative conclusions, and so Fig. 1 shows the results we obtained using different subsets of observations and different amounts of 'smoothing' to bracket the range of possible answers. The existence of a large emission in Southeast Asia, of the order of 1 Pg C, appears in all combinations of the estimation techniques and data used. So, although the figures produced by Page *et al.* appear to be extraordinarily large, they are supported by independent approaches.

But why were the fires in Indonesia so serious? It took a combination of climate change — specifically, drought, caused by the 1997–98 El Niño event — and changes in

land use brought about by human agency. Drought-induced fires are common in the tropics, and also affect other parts of Australia and Amazonia. But in Indonesia their intensity and duration, and the damage wrought, were all the more severe because small clearings and channels associated with logging operations allowed the vegetation and soils to dry out, making them more susceptible to burning. Because of the thickness and high carbon density of the peat, the carbon emissions from the fires were extremely high.

This all points to a general challenge for those studying the carbon cycle. Detecting the subtle and fine-grained effects of land-use change, such as that occurring in Indonesia and elsewhere, requires satellite imagery of 30-m resolution. This is far beyond the reach of today's models of the terrestrial carbon cycle, which have 50-km resolution, at best. Future work to understand and predict the system's behaviour must take account of how land use can be a factor in fire and other disturbances. Changed land use,

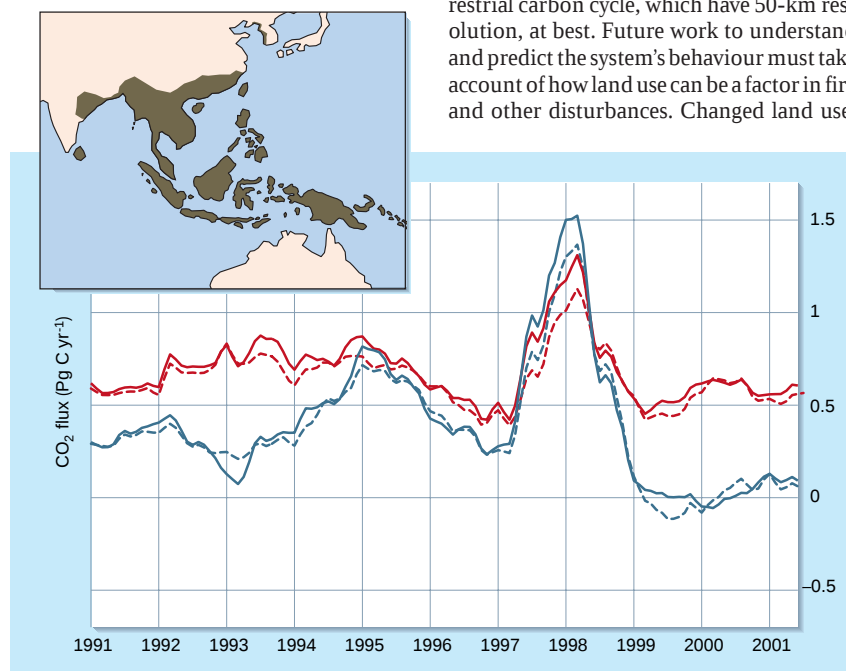


Figure 1 The CO₂ pulse of 1997–98 from Southeast Asia. Page *et al.*¹ estimate that, in releasing 0.8–2.6 Pg C during this time, wildfires in Indonesia were responsible for much of the increase in levels of atmospheric CO₂. Atmospheric modelling supports this view, as depicted here. Our global analysis reveals a large emission in Southeast Asia, and is the average of ten models³, each run with two data sets and different degrees of smoothing. Red lines show data⁶ from 97 monitoring stations; blue lines are data from a subset of 90 stations, omitting seven in the South China Sea. Dashed and solid lines show different degrees of smoothing of the variability in ocean fluxes. The land areas in Southeast Asia defined in the atmospheric model are shown by the shaded area of the inset map. The Indonesian fires were probably only part of the response of the global carbon cycle to the El Niño of that period, as other tropical land regions also show high emissions during 1997–98 in this modelling analysis.

and the subsequent environmental perturbation, can help to bring about both abrupt emissions of CO₂ and long-lasting alterations in ecosystem structure and function.

The need to understand such abrupt change is pressing. For decades, carbon-cycle researchers have analysed observations of atmospheric CO₂ in terms of processes such as ocean CO₂ uptake, and photosynthesis and respiration by land plants. These processes are assumed to operate continuously and relatively smoothly in time and space. Analytical approaches such as inverse modelling tend to assume that the same processes are operating at all times⁴. Despite the advent of so-called 'dynamic global vegetation models', modelling remains dominated by simulations of photosynthesis and respiration, with only primitive representations of combustion⁵.

The results of Page *et al.*¹ make it clear that abrupt events can have an appreciable effect on the carbon cycle. The fires in Indonesia

were not only episodic in time, they occurred on a relatively small part of the Earth's land surface. Most observing systems and modelling strategies assume that, to affect the carbon cycle, processes must occur over thousands of square kilometres or more. But especially in areas of high carbon density, catastrophic events affecting small areas can evidently have a huge impact on the global carbon balance. ■

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Vision

What is a naked retina good for?

Michael F. Land

The crab *Bythograea thermydron* has eyes that consist of large naked retinas. Studies of the crab's larval forms suggest that these eyes are a specific adaptation to the dim environment of oceanic volcanic vents.

Along the mid-ocean ridges of the Atlantic and Pacific are volcanic vents from which water emerges at temperatures of up to 350 °C, more than three times the boiling point at the surface. This heat, and the minerals that emerge with the water, supports a diverse fauna, including a number of crustaceans with very unusual eyes. On page 68 of this issue, Jinks and colleagues¹ take our understanding of these optical systems further, and their results provide clues to the light environment around vents.

The shrimp *Rimicaris exoculata* was the first vent crustacean with unusual eyes to be described². Here, the optics of the compound eye, which allow images to be resolved, have disappeared and been replaced by an extended naked retina much larger than the eyes of related species. But did this peculiar arrangement evolve through degeneration, caused by the absence of light in the deep ocean (the vents are several kilometres below the surface)? Or is it a specific adaptation to the radiation environment around the vents (Fig. 1)? One approach to this problem is to look at the development of these eyes as the animal changes from larva to adult. If the eye starts out as a normal compound eye, then this strengthens the argument that the later naked retina is a specific adaptation to the vent environment: otherwise, why go to the trouble of making

one structure, then unmaking it and effectively replacing it with something else?

This is the approach that Jinks *et al.*² have taken in their studies of the crab *Bythograea thermydron*. Adults of this species inhabit vents and have naked retinal structures similar to those of *Rimicaris*. Crucially, several other stages in the life cycle are also known, including early ('zoea') larvae, which live not in vents but as deep-sea plankton. Studying

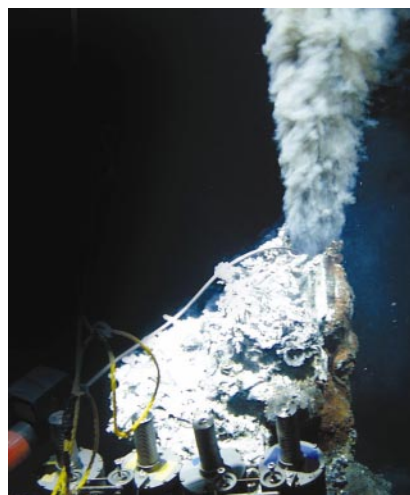


Figure 1 Deep-sea volcanic vent — home to species such as the crabs studied by Jinks *et al.*¹.

crabs reared from egg-bearing females, the authors found that these planktonic larvae have normal apposition compound eyes, which progressively lose their optics during development and turn into the naked, rather amorphous, retina of the adult.

The pigments in the eye that respond to light also change: they are maximally sensitive to blue light (447 nm) in the planktonic zoea larvae; to blue-green light (479 nm) in the later megalopa larvae, which already inhabit vents; and finally to longer-wavelength blue-green light (489 nm) in the adult eye. What is striking here is that this change is the opposite of what one might expect. Surface light has most energy in the green region of the spectrum, but, with increasing depth, scattering and absorption reduce the transmitted light to a narrow waveband in the blue, around 470 nm. Visual photopigments usually reflect this change.

So Jinks and colleagues' finding that *Bythograea* shows the opposite trend with depth suggests that there might be something interesting about the composition of the light — such as it is — around the vents. It was originally thought that there was just enough short-wavelength energy in the black-body radiation from the vent water itself to provide a few photons that would allow a green-sensitive pigment to detect something. But more recent measurements suggest that there is still more light in the visible range³. Sulphide oxidation can cause chemiluminescence in the visible range, and there are several other more exotic physical phenomena that might also contribute. Whatever the origin of vent light, however, it seems that there is enough for rudimentary vision with a non-resolving retinal structure.

This raises another question: why have an eye with no optics? Clearly, a bare retina cannot determine the direction of light to better than the nearest hemisphere — in front of the animal or behind it. The gain, in abandoning the machinery of resolution, is sensitivity.

The amount of light available to a detector from a given source of light is proportional to the area of the aperture of the detector, and the size of the cone (solid angle) over which light is received⁴. In a shore crab, the area of the aperture of each eye facet is about 0.001 mm², and the solid angle over which the receptors accept light is about 3 square degrees. For adult *Bythograea*, if the whole eye is viewed as a single detector, the aperture area is about 1 mm², and the acceptance cone is a hemisphere (20,626 square degrees). The overall sensitivity ratio between the naked retina and the proper eye is then about 7 million to 1. If what is required is a non-directional photocell, used for example to establish proximity to a vent, then a naked retina makes very good sense.

Naked retinas are not confined to vent

animals. The benthic fish *Ipnops murrayi* has the surface of its flattened snout covered in naked retinas, and many crustaceans from deep water seem to have converged on the same drastic strategy for finding the vague direction of very dim light. Certainly in *Bythograea*, and probably more generally, it seems that such eyes are not degenerate but are well adapted to the prevailing light conditions. ■

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Astronomy

Twinkle, twinkle, neutron star

Cole Miller

Neutron stars, as the name suggests, are mostly made of neutrons. But the cores of these tiny, dense stellar leftovers might conceal new states of matter, including strange matter. The light from these stars holds the key.

Neutron stars, formed during the death throes of massive stars, are among the most exotic objects in the Universe. They are the size of a city but typically contain at least 40% more mass than our Sun. As a result, their core density is several times the density of an atomic nucleus. In principle, this means that neutron stars may be our only window on the properties of matter beyond nuclear density. But in practice, this window has been hard to open, because spectroscopic information — the cornerstone of astronomical data — is exceedingly difficult to obtain for neutron stars. As Cottam *et al.*¹ report on page 51 of this issue, and as Sanwal *et al.*² discuss in the *Astrophysical Journal*, this situation may be about to change.

The properties of extremely dense matter have long been a focus of research in nuclear physics. The most mundane possibility is that matter simply becomes very neutron-rich at high densities. Even then, the extraordinary densities in neutron stars lead to the onset of phenomena such as superfluidity and superconductivity — the latter at a transition temperature of around 10^9 K, making neutron stars the true high-temperature superconductors of the Universe. But it has also been suggested that in neutron-star cores, unique forms of matter may dominate. These include Bose–Einstein condensates of subatomic particles such as pions and kaons; enormous ‘bags’ of the elementary particles known as quarks; and so-called strange matter.^{3,4}

Learning about dense matter from neutron stars is challenging because observations only provide indirect information. One approach is to constrain the values for the mass and radius of individual stars. If high-density matter is especially compressible (as it will be if exotic components are present), the star will be comparatively small for its mass. The presence of exotic matter

also lowers the maximum stable mass for a neutron star. For example, data from the satellite-based Rossi X-ray Timing Explorer suggest⁵ that the neutron star 4U1820–30 has a mass 2.2 times that of the Sun, which is probably too high for it to contain exotic matter; but the complicated properties of this source make the mass estimate highly uncertain. If a neutron star has a mass of around 1.4 solar masses, the range of theoretically allowed radii^{6–8} is roughly 7–15 km, although this range is more restricted if one considers only standard models based on modern nuclear scattering data⁹. Although the masses of many neutron stars have been estimated from observations of binary orbits, measurements of their radii have proved elusive.

How can astronomers measure the size of an object that is probably just 10 km in radius but more than 10^{16} km away? One solution is high-resolution spectroscopy. Each type of atom or molecule radiates at particular energies, and so can be identified in the spectrum of light from a star. The effects of gravity cause the observed energies of the spectral lines to be shifted to lower values, by a factor of $1/(1+z)$, where z is called the redshift. This redshift depends on the ratio of the star's mass to its radius, and so measuring the shift in spectral lines provides indirect information about the stellar radius. Returning to the example of a neutron star with a mass of 1.4 solar masses, radii of 15 km down to 7 km correspond to $z = 0.18$ – 0.56 ; $z = 0.30$ for a canonical radius of 10 km. But X-ray instruments did not have the sensitivity and resolution needed to detect such redshifts, until now.

The new results have been achieved through the remarkable spectroscopic capabilities of the space-borne Chandra X-ray Observatory and X-ray Multi-Mirror (or XMM–Newton) instruments. Sanwal *et al.*² report Chandra observations of the young

isolated neutron star 1E1207.4–5209. They have found two absorption lines in the spectra, at energies of 700 and 1,400 eV (electron volts), which they interpret as the signature of singly ionized helium in a strong magnetic field. The implied redshift is 0.12–0.23. As well as gravity, the magnetic field (around 10^{12} gauss) has a large effect on the line energies. But because the field strength is not known accurately, Sanwal *et al.* cannot be sure that they have correctly identified the lines, and neither can they conclusively rule out other interpretations (such as those based on cyclotron features).

This is where the results of Cottam *et al.*¹ come into play. They obtained XMM–Newton observations of EXO0748–676, a neutron star that is accreting gas from a lower-mass star. For various reasons, the neutron stars in such systems are thought to have surface magnetic fields of around 10^7 – 10^9 gauss. Although staggering by terrestrial standards (the Earth's magnetic field is just 1 gauss), these fields are too weak to have a significant effect on atomic spectra at energies higher than 100 eV. So it is easier

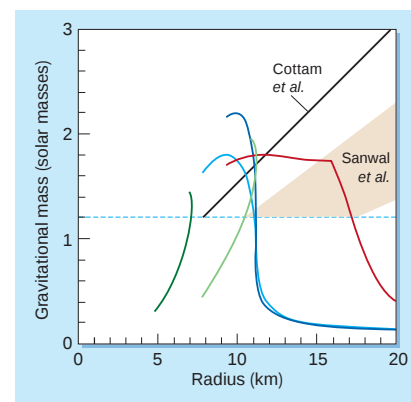


Figure 1 The structure of neutron stars. The gravitational mass and the radius of a neutron star are both related to the ‘redshift’ that can be measured in the spectrum of light emitted by the star. The range of possible values for mass and radius is shown for various models of the neutron-star interior: stars containing strange matter (light- and dark-green curves); stars made of normal matter (light and dark blue); and stars whose cores become a Bose–Einstein condensate of pions if their mass is higher than 1.8 solar masses (red). The blue dashed line represents a conservative lower limit, at 1.2 solar masses, for the mass of a neutron star formed from a supernova. From the redshifts measured for two different neutron stars, Cottam *et al.*¹ and Sanwal *et al.*² have derived the allowed values of mass and radius (black line and shaded region respectively) and find that they are not consistent with some models of strange-matter stars. The mass of neither source is known at present; accurate mass measurements would clearly strengthen the constraints considerably.

to determine the redshift from this star's spectrum than it is from that of a strong-field neutron star.

Cottam *et al.* observed EXO0748–676 during a series of 28 X-ray bursts — the flashes from thermonuclear fusion that occur when sufficient hydrogen and helium has piled up on the star's surface. They found three strong spectral lines, which they identify as the signatures of 25-times-ionized iron (Fe xxv in astronomical nomenclature), Fe xxv and O viii. The inferred redshift for all three is 0.35, which defines a range of possible values for the mass and radius of this star (Fig. 1). Such identifications should always be handled with care, because there are in principle many atomic species and many transitions to check. But Cottam *et al.* make a good case that these are the most probable sources of these lines. If the identifications are correct, the redshift is exactly in the range expected for a star made of normal neutron matter, and doesn't fit the models for the most compact strange-matter stars⁶, although there is still room for exotic components (Fig. 1).

The importance of these results inspires caution. It would be good to see lines measured with self-consistent redshifts from

more neutron stars, to guard against the possibility that the lines have been misidentified in these two sources. Moreover, constraints on models for high-density matter would be strengthened considerably if both the mass and gravitational redshift were measured for other neutron stars. In any case, high-resolution instruments such as Chandra and XMM–Newton are dramatically fulfilling their promise as sensitive probes of matter in extreme environments. ■

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Protein folding

With a little help...

Charles L. Brooks III

Experiments and computer simulations are converging in their exploration of the timescales on which protein folding occurs. Such developments are a promising way forward in molecular biophysics.

From a cast of thousands, and the experiments of a few, a new combination of computer simulation and experiment to investigate protein folding has emerged. This work, described by Snow *et al.* on page 102 of this issue¹, shows that the key events in the folding of a small artificial protein can be directly examined by atomic-level computer simulation. Moreover, the results are in general agreement with experiments performed under analogous conditions.

Snow *et al.* have brought the distributed computing machinery embodied in the Folding@home project^{2,3} to bear on the direct simulation of folding of small, though conformationally rich, polypeptides. The polypeptides are based on a specially designed mini-protein named BBA5 — which, for all its small size, nonetheless adopts a canonical native fold, the α -helix and β -hairpin. The authors look at the folding of BBA5 mutants (that is, proteins with sequences that differ from BBA5 by substitutions of one or two residues), and show that the folding occurs on a timescale of 1–10 μ s at temperatures

near 300 K. The general agreement between experiment and simulation for this system suggests that current methods for describing atomic interactions by computer simulation (with 'force fields' and molecular-dynamics methods) are adequate for exploring the timescales and energies of folding.

Folding is a highly complex, structure-organizing process, in which a chain of amino acids passes through several stages before the protein concerned — say, an enzyme — reaches its final folded and active form. Many small proteins adopt their folded state from a diverse set of less structured conformations by crossing a single barrier (transition state) in a nearly all-or-none, two-state fashion on timescales ranging from a few microseconds to milliseconds and longer. Progress in experimental methods during the past few years provides the tools to explore folding processes on the fastest of these timescales⁴. On the other hand, even the fastest timescales are very long compared with routine times accessed in molecular-dynamics simulations of proteins.

The problem, then, in computer simula-



100 YEARS AGO

The Health Department of the City of London has had a number of samples of ice-creams bacteriologically examined. A large proportion of the samples were found to be unsatisfactory; in several micro-organisms were very numerous, while in some virulent organisms of the *Bacillus coli* type were present; one contained pyogenic organisms and produced abscesses in guinea-pigs, and another contained an anaerobic organism, perhaps the bacillus of malignant oedema. Many of the ice-creams from which samples were examined had set up gastro-enteritis in boys employed by the Post Office.

ALSO...

New fields for research are continually opening up; the last illustration of this is the discovery by Prof. G. Elliot Smith that it is possible to map the convolutions of the brains of non-mummified ancient Egyptians. The brain is naturally preserved in the vast majority of the bodies in Egyptian cemeteries from predynastic to recent Coptic, the favourable conditions being burial in dry soil and removal from all direct access to the air... In a memoir, which will be published in a short time, he intends to give a full account of the structure of the brain in the predynastic and protodynastic Egyptians. From *Nature* 6 November 1902.

50 YEARS AGO

The Nobel Prize for Physiology and Medicine for 1952 has been awarded to Prof. Selman Abraham Waksman... for his discovery of streptomycin, the first effective antibiotic against tuberculosis. Prof. Waksman was born in 1888 in Priluka, a small town in the Ukraine, emigrated to the United States in 1910 and became a naturalized citizen there in 1915. The whole of his scientific life since 1911 has been spent at Rutgers University and has been devoted to the study of microbiology, and particularly to that group of soil micro-organisms which are frequently spoken of as ray fungi and belong to the genus *Actinomyces* or *Streptothrix*. Following the discovery of penicillin, which is fully effective only against Gram-positive bacteria, Waksman and his collaborators began, in 1939, a systematic search for an antibiotic active against Gram-negative bacteria and found it, in 1944, in streptomycin, a metabolic product of *Streptomyces griseus*... They also showed that streptomycin is highly active *in vitro* against *Mycobacterium tuberculosis*.

From *Nature* 8 November 1952.

tions of protein folding, is to 'beat Boltzmann' — that is, beat the odds of arriving at the transition state for folding and unfolding while retaining some information about the timescales of reaching this point. One answer to this problem biases the energies of protein conformations, using methods called umbrella sampling, to restrain the regions of conformational space the system explores, and then reconstructs the unbiased energy landscape using theoretical techniques from statistical mechanics. This approach has been successfully applied to several different protein structures, lending insights into protein-folding characteristics such as the manner in which protein topology dictates the folding mechanism, the direct role that the solvent (water) can play in mediating the later stages of protein folding, and a host of other processes⁵.

Beating Boltzmann can also be accomplished by looking at the reverse process and increasing the probability of arriving at the unfolding transition state through the use of high temperature. By coupling the protein-solvent system to a high-temperature bath (typically well above the standard boiling point of water), molecular-dynamics simulations can be performed over timescales of many nanoseconds to observe the protein denaturing. Information about the mechanism and timescale for protein unfolding can be directly gleaned from such simulations, although experimental unfolding rates must be extrapolated to the temperatures of the simulations to make direct comparison⁶. Both of these approaches are yielding insights into the problems of protein folding and unfolding. But neither permits the direct observation of folding dynamics under folding conditions.

To explore folding directly, as Snow *et al.*¹ have done, one must overcome the 'Boltzmann waiting time' required by individual molecular trajectories to sample transition-state conformations and proceed into the energetic 'basin' of native folds. A clever way of doing this, employed by Snow *et al.*, is to use the statistical nature of the distribution of these waiting times. By considering (tens of) thousands of trajectories, some protein chains will be in conformations near the transition region, and will proceed rapidly to the native fold. Monitoring the fraction of such trajectories and the timescale required, on average, for these protein molecules to reach the native fold leads to an estimate of the mean folding time. This is simply constructed as the ratio of the length of an individual trajectory to the fraction of trajectories that folded in that time. Snow *et al.* examined 7,500–9,000 trajectories for durations of 20 nanoseconds for each of two mutants of BBA5, and observed some 100 folding events. The resulting estimates of the folding time were in good agreement with their experimental findings.

To achieve the amount of sampling of individual trajectories needed for Snow and colleagues' studies, a highly distributed and loosely coupled network of volunteer computers, exploiting the tremendous resources of personal computers connected to the Internet, was used. Because each folding trajectory is independent of the others, work to compute the molecular dynamics of folding can be parcelled out to the volunteers' home computers using the web. The software developed by Snow *et al.* manages this task and assembles the computed properties for analysis of folding. Distributed computing systems such as Folding@home are an enormous resource for exploring scientific questions such as protein folding, and provide a new way of simulating complex biological processes. They also represent unique social experiments, permitting the public at large to be directly involved in investigations ranging from a search for drugs to combat AIDS⁷ to the quest for intelligent life beyond the Earth^{8,9}.

The future will see a continuing expansion of the worldwide resources exploited by

Folding@home as personal computers and networks become faster. New problems will be examined and new questions answered. As for protein folding, larger and more realistic systems will be required to truly advance our understanding of this complex process. Whether this is possible with current models remains to be demonstrated — with a little help from our friends. ■

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Plant ecology

Express delivery by bat

Peter D. Moore

The seeds of plants need to be dispersed to locations where they can survive and grow. In the deserts of Mexico, it seems that a species of bat is the dispersal agent of choice for a giant cactus.

Eating fruit is a means of making a living for many mammals and birds. And from the plant's point of view, frugivores may provide an answer to the vexed problem of seed dispersal. But some fruit-eaters are more effective at the job than others, in terms of distance covered, quantity consumed and the quality of the locations in which seeds are eventually deposited. Some frugivores, in other words, are worthy of more encouragement than others. Writing in the current issue of *Ecology*¹, Godínez-Alvarez, Valiente-Banuet and Rojas-Martínez come to the conclusion that a species of bat is the best option for dispersing the seeds of the giant columnar cactus *Neobuxbaumia tetetzo*, found in the Tehuacan Valley of Mexico. The authors' work reveals a fine example of coevolution in action.

It is in the evolutionary interests of an individual plant to leave as many copies of its genes in the next generation as possible. This involves adequate seed production, but it is even more important to ensure that seeds are transported to sites where their survival and success is assured². The most efficient frugivorous dispersal agent will consume the fruit readily, will have a gut in which the seed is not damaged (and where its germination poten-

tial might even be enhanced), will travel away from the parent plant after fruit consumption, and will defecate in a location that is suitable for seed germination, establishment and survival. The evolution of such dispersal systems therefore involves the selection of the most appropriate agent and the modification of the fruit to suit the needs of that agent.

In the Tehuacan Valley of central-southern Mexico, the desert vegetation is dominated by a giant columnar cactus, *Neobuxbaumia tetetzo* (Fig. 1). However, the vegetation is heterogeneous, areas of cactus being interspersed with patches of scrub consisting largely of *Mimosa luisana*³. The vegetation forms a dynamic system in which the cactus seedlings survive only in the shade of the *Mimosa* bushes, but as the cacti grow, so the bushes die and the mature cacti form pure stands. The eventual death of an ageing cactus is then followed by invasion of shrubs, and the cycle continues.

The cycle is maintained because of the germination and establishment requirements of the young cacti. The seedling stage is the most vulnerable phase of the plant's life, and studies have shown⁴ that giant cacti may need the shade of other plants in their very early stages of growth to protect them



Figure 1 **Desert delights: the cactus *Neobuxbaumia tetetzo*. New work¹ suggests that, of a variety of frugivores, the lesser long-nosed bat is the best option for dispersing the seeds of this plant.**

from the desiccating effects of sunlight. The seed germination of *Neobuxbaumia* is very poor in open locations and is highest in the shade of *Mimosa*³. So the cactus needs its dispersal agents to deposit its seeds near *Mimosa*.

Several birds and mammals have been observed taking the fruits of the cactus, including the coyote, grey fox, cactus wren, grey-breasted woodpecker, house finch and lesser long-nosed bat¹. Godínez-Alvarez and colleagues¹ assessed the value of each of these consumers to the plant, considering the quantity of seeds ingested, seed survival in the gut, and the quality of the sites where deposition takes place. The quantity factor was estimated by 'mist-netting' the visitors to selected individual cacti during the day (birds) and at night (bats). Seed-removal experiments were also set up, which recorded seed losses during the day and the night. Coyotes and foxes fed mainly on fruits that had fallen to the ground. The most frequent visitors to the cacti were the bats, and these were also the most voracious fruit consumers, so bats win out on the quantity front.

Seed survival in the gut was zero for the house finch, so this species must be regarded as a seed predator rather than a potential dispersal agent. For other birds and for bats, however, seed germination was high after deposition. The location of deposition is the final factor to be calculated, and the different

organisms varied in their postprandial behaviour. Some birds, such as the woodpecker, showed little inclination to perch in the *Mimosa* shrubs after fruit consumption, whereas others, such as the cactus wren, had a 43% probability of using the shrubs for resting and defecation. For the bat, however, the probability of depositing seeds among the shrubs was as high as 72%.

The research team was able to put all of

these factors together and model the likely plant-demographic outcome of fruit consumption by each of the various frugivores. On the basis of the high frequency of visits, the high fruit consumption, the good seed survival in the gut and the strong likelihood of deposition in an appropriate location beneath *Mimosa* bushes, bats showed a very strong lead in the role of seed carrier for the cactus. Lesser long-nosed bats, it seems, play a key role in the reproductive biology and the population dynamics of this plant.

If the bat is indeed such an effective dispersal agent, one might expect features to evolve in the plant that enhance fruit consumption by bats rather than by alternative visitors. Two such adaptations are apparent. The flesh of the fruit of *Neobuxbaumia tetetzo* is colourless, whereas the fruits of *Carnegiea gigantea*, another giant columnar cactus in the region, have bright red flesh that is particularly attractive to birds. So *Neobuxbaumia* is evidently not targeting birds. The second feature is that the fruits of *Neobuxbaumia* ripen during the night.

Coevolution between plant and seed-dispersal agent is evidently under way here, although there is not as yet a complete interdependence. Indeed, it may be in the plant's interests to maintain a dispersal back-up, in the form of less efficient birds. This work¹ does, however, provide a new glimpse into the demographic consequences for the plant of seed-carrier selection. ■

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Condensed-matter physics

Magnetic frustration squeezed out

Peter Schiffer

Atomic spins in a 'frustrated' magnet cannot simultaneously minimize the energies of their local interactions: they fluctuate continuously, even at very low temperatures. But under pressure, they line up in an ordered way.

While we all occasionally experience frustration in our lives, the consequences of extreme frustration have become an increasingly active area of magnetic materials research. Magnetism arises from the atomic spins in a material, and 'frustration' occurs when the interactions between the spins are in close competition with each other. In magnetic materials, frustration leads to a variety of cooperative spin states with evocative names

such as 'spin glass', 'spin liquid' or 'spin ice' — all originating from the delicate balance of frustrated spin-spin interactions. On page 54 of this issue, Mirebeau *et al.*¹ demonstrate just how precisely nature has balanced these interactions.

If we define frustration as a close competition between different interactions, then the most strongly frustrated physical systems are those in which the interactions allow the components to be arranged in many

equivalent minimum-energy configurations. In the language of condensed-matter physics, a strongly frustrated system has a high density of low-energy excitations, or 'soft modes', that correspond to fluctuations between these energetically equivalent configurations². Strong frustration of this sort can be most easily studied in geometrically frustrated magnets, where the spatial arrangement of atoms results in competing interactions among the atomic spins.

A simple example of local geometrical magnetic frustration is the arrangement of three identical spins on an equilateral triangle (Fig. 1). The spins are constrained to point either up or down, and the energy of interaction between any two spins is minimized if the two point in opposite directions. The individual interaction energies of all three pairings on the triangle compete and cannot be minimized simultaneously, but the total energy of the triangle is the same for any of the six different spin configurations.

Experimental studies have shown that geometrical magnetic frustration and the associated statistical mechanics can lead to a variety of unique behaviours in materials with a range of frustrating geometries (Fig. 2). Some of the most interesting of these materials have the 'pyrochlore' geometry, in which the magnetic ions are located on a three-dimensional lattice of corner-sharing tetrahedra. Experiments on frustrated pyrochlores have shown a wide range of novel physical phenomena: Berry-phase effects on the anomalous Hall effect³; glassy behaviour in the presence of minimal disorder^{4,5}; statistical mechanics equivalent to that of protons in ice^{6–8}; and 'cooperative paramagnetic' behaviour⁹, in which the spins do not order themselves but continue to fluctuate at temperatures much lower than the energy scale of the interactions between them and form a 'spin liquid'. These spin-liquid phases^{10–12} result from an energy landscape that has a continuum of energetically equivalent states, with low or nonexistent barriers between them. Such a landscape allows the spins to fluctuate between different, correlated configurations at low temperatures, despite being coupled to neighbouring spins with an interaction energy much greater than the temperature.

Mirebeau *et al.*¹ report a study of $\text{Tb}_2\text{Ti}_2\text{O}_7$, a frustrated pyrochlore compound in which the Tb^{3+} ions have magnetic moments that are several times that of a free electron. $\text{Tb}_2\text{Ti}_2\text{O}_7$ is a spin liquid at low temperatures, with no evidence of long-range magnetic order down to temperatures of 70 mK, despite a spin–spin interaction (associated with dipolar and exchange coupling) with an energy scale greater than 10 K. The authors examined $\text{Tb}_2\text{Ti}_2\text{O}_7$ through the technique of neutron diffraction, which elucidates the microscopic spin configuration. Unlike most other neutron-

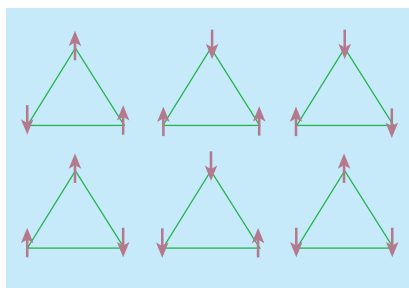


Figure 1 Frustrated spins. In this example of geometrical magnetic frustration, three identical spins arranged on a triangle are constrained to point either up or down, and each spin interacts antiferromagnetically with each neighbour — in other words, the energy of interaction between two spins is minimized if the two point in opposite directions. The individual interaction energies of all three spin-pairings cannot be minimized simultaneously, but the overall total energy is the same for any of the six configurations shown.

diffraction studies, however, they work at the extremes of low temperature and high pressure — up to nearly 10^{10} Pa, almost 100,000 times atmospheric pressure. These conditions stretch the limits of neutron-scattering technology, as such high pressures can only be obtained in so-called anvil cells that require the neutrons be channelled to and from a tiny sample volume.

Despite the experimental difficulties, Mirebeau *et al.* were able to obtain high-quality data which, surprisingly, show that the application of pressure produces sharp peaks in the diffraction pattern below a transition temperature of about 2 K. The authors attribute these peaks to the formation of long-range order among a sizeable fraction of the spins. Such long-range ordering would indicate that, under pressure, the spins find a particular state into which they settle at low temperature, rather than continuously fluctuating between numerous equivalent configurations in the spin liquid. In other words,

the application of pressure eliminates the precise balancing of frustrated interactions between the spins that allowed the spin liquid to exist.

No such pressure-induced ordering has previously been observed in a geometrically frustrated spin liquid. These results are particularly interesting because the pressure is applied almost isotropically to the sample, through a surrounding transmission medium. Thus the lattice is uniformly squeezed rather than distorted in the pressure cell, and the geometrical frustration might have been expected to remain. Ordering of a geometrically frustrated spin liquid has previously been seen in an applied magnetic field¹³, but the perturbation in that case is anisotropic and so would be expected to reduce the symmetry and the number of accessible spin configurations. In the work of Mirebeau *et al.*¹, a uniform compression of the lattice would not be expected to induce such anisotropy, and the resulting long-range order hints at the extreme delicacy of the frustrated balance of spin–spin interactions.

There are still many open questions concerning the fundamental nature of spin-liquid phases in geometrically frustrated magnets. For classical spins, the spin-liquid phase should persist down to zero temperature⁹, and the constituent spins in many of these materials are so large that they would typically be treated classically. Experimental observations of these phases down to low temperatures^{11–15}, however, suggest that quantum-mechanical fluctuations might be important. Although the pressure-induced transition observed by Mirebeau *et al.*¹ seems to be of first order, their work raises the possibility of novel quantum-phase transitions from the spin-liquid state as a function of applied magnetic field or pressure. As more geometrically frustrated magnetic materials are discovered and examined in greater detail, the new low-temperature phenomena that emerge will offer a unique window on the fundamental nature of

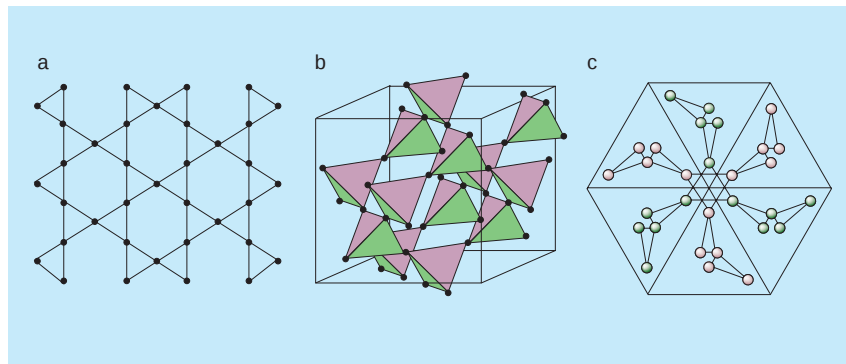


Figure 2 Lattices of frustration. Examples of different geometrically frustrated magnetic lattices that have been realized in magnetic materials: a, the two-dimensional kagomé lattice found in $\text{SrCr}_{12-x}\text{Ga}_x\text{O}_{19}$ (ref. 14); b, the three-dimensional pyrochlore lattice found in $\text{Tb}_2\text{Ti}_2\text{O}_7$, the compound studied by Mirebeau *et al.*¹; and c, the three-dimensional garnet lattice observed in $\text{Gd}_3\text{Ga}_5\text{O}_{12}$ (refs 13, 15).

collective fluctuations in strongly correlated systems.

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Astronomy

Close encounters of the tidal kind

Tommy Wiklind

Between the Magellanic Clouds, in a region swept with tides of gas, stars are forming. The detection of carbon monoxide shows gas is condensing, and further observations may reveal the ultimate fate of the clouds.

As giant galaxies merge, small galaxies may be born in the gaseous debris thrown out from the collision. This kind of ‘tidal debris’ is also seen in our own neighbourhood, as the Milky Way and two nearby galaxies, the Magellanic Clouds, act out a gravitational tug-of-war with each other. Writing in the *Monthly Notices of the Royal Astronomical Society*, Muller *et al.*¹ announce their detection of cold, dense molecular gas in the Magellanic tidal debris, signalling the birth of new stars in this inhospitable place and possibly offering a unique window on galaxy formation.

The Magellanic Clouds — the Large

Magellanic Cloud (LMC) and the Small Magellanic Cloud (SMC) — are dwarf galaxies and gravitationally bound satellites of our own, much larger, Milky Way galaxy. These clouds have endured a rather violent life over the past 1.5 billion years or so, which has left a number of tell-tale signatures: a long, trailing stream of neutral atomic-hydrogen gas, stretching almost 100° across the southern sky; a ‘leading arm’ (a second stream of gas that seems to lead the motion of the clouds) also of neutral hydrogen gas; and a gaseous bridge connecting the LMC and SMC. These features appear clearly in a recent all-sky map (Fig. 1) derived from a well-known atomic-

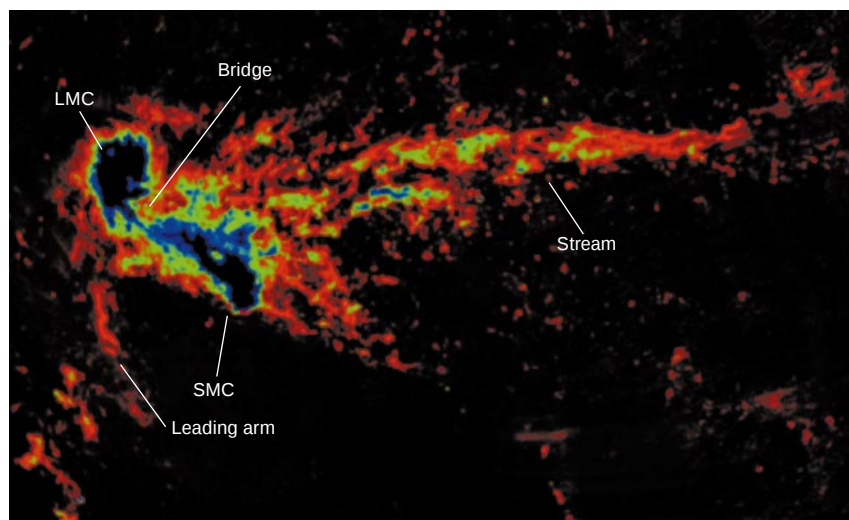


Figure 1 Evidence of a violent past. The distribution of atomic-hydrogen gas in the southern sky is captured in this image from the Parkes radio telescope in Australia². Two concentrations of gas form the Large and Small Magellanic Clouds (LMC and SMC), and behind them the long tail of the Magellanic stream stretches more than 100° across the sky. Two hundred million years ago, a close encounter between the LMC and the SMC led to the formation of the Magellanic bridge between them. Muller *et al.*¹ have detected cold, dense molecular gas in the bridge, which shows that stars are forming in this region. (Image adapted from ref. 2.)

hydrogen emission line (the 21-cm line) using the Parkes telescope in Australia^{2,3}.

An explanation for these features has emerged through computer modelling of a three-body gravitational interaction between the LMC, the SMC and the Milky Way⁴. The long Magellanic stream and the leading arm are, in this model, the result of a close encounter between the SMC and the Milky Way about 1.5 billion years ago. The gas making up the stream comes from the inner regions of the SMC, but the stream does not seem to contain any stars. The bridge connecting the two Magellanic Clouds is thought to result from a particularly close encounter between the LMC and the SMC a ‘mere’ 200 million years ago. Again, the gas is believed to have been pulled from the SMC, the less massive of the two galaxies. But in contrast to the stream, the bridge does contain stars, even stars much younger than 200 million years. So these stars cannot have formed before the encounter between the two Magellanic Clouds, but instead must have formed in the bridge itself.

Star formation in galaxies is directly associated with the presence of cold, dense molecular gas. As stars are being born in the bridge between the Magellanic Clouds, it is expected that molecular gas exists there, in cohabitation with the more diffuse and dispersed atomic gas. The main constituent of a molecular cloud is molecular hydrogen, which is a poor emitter of photons because of its symmetrical structure. Molecular gas is therefore usually observed through its rarer constituents, such as carbon monoxide (CO) and other species. For these tracer molecules to be present the gas must be enriched in ‘metals’ (in astrophysical language, anything heavier than helium is referred to as a metal). The gas in the bridge is believed to have been drawn from the SMC, which is rather metal-poor, with metal content only around 10% that of the Sun. In fact, measurements indicate that the bridge contains a lower amount of metals than the SMC, so there are considerably fewer C and O atoms to make up observable CO molecules.

Nevertheless, Muller and colleagues set out to search for the telltale CO emission in selected regions of the Magellanic bridge, using both the 22-m MOPRA telescope in Australia and the 15-m SEST telescope in Chile. Out of six candidate regions, they had time to survey only one, but were rewarded with a successful detection. The emission is associated with a region where young stars, dust and atomic hydrogen are all mixed together. But, compared with the SMC, the molecular emission is weaker and the inferred gas velocity has a smaller spread of values. Moreover, atomic gas in the SMC has two distinct velocity components, only one of which has associated CO emission⁵. But the CO emission in the bridge seems to be

associated with the other velocity component, implying that it condensed from atomic gas *in situ*, rather than being extruded as molecular gas from the inner regions of the SMC itself.

The fate of the gas and stars in the Magellanic bridge is presently unknown. Will it fall back into either of the Magellanic Clouds? Or will it be absorbed into the Milky Way? A third, though more remote, possibility is that the bridge will form a more or less independent system — the Very Small Magellanic Cloud (VSMC). To assess the likelihood of these possibilities, the total mass of the bridge, as well as its kinematics, must be measured and this is best done by observing its atomic and molecular gas components. Should the third scenario indeed be the final outcome (before the Magellanic systems merge with the Milky Way, which is the ultimate outcome), it would mean that a small galaxy has been born right before our eyes.

This would indeed be a remarkable opportunity: one of the outstanding problems in modern astrophysics is to understand how galaxies form. The most successful model of galaxy formation so far has galaxies surrounded by haloes of mysterious, cold dark matter (CDM) — the unseen, 'missing' matter that pervades our Universe; matter as we know it, in the form of stars and gas, is just a small part of the entity that we call a galaxy. In this model, small galaxies form first, dominated by CDM, and are then assembled into larger and larger complexes through gravitational attraction and subsequent merging, following a scheme of so-called hierarchical clustering.

According to the CDM model, in early times our Universe contained relatively large numbers of small galaxies, and this certainly seems to have been the case. The present-day Universe, on the other hand, should contain large galaxies that occasionally grow by 'swallowing' one of the surviving small galaxies. This also seems to be in accordance with observations and the Magellanic system is an example of this process.

But should the Magellanic bridge become free of the LMC and SMC, it would constitute yet another method of galaxy formation. In fact, tidal-debris formation of small galaxies is known to occur in the Universe. Dwarf galaxies formed during violent gravitational interaction between large galaxies — Tidal Dwarf Galaxies, or TDGs — are relatively common, and some of these TDGs are known to contain both stars and molecular gas in a manner similar to the Magellanic bridge⁶. There are even cases where tidally created galactic entities seem to contain only gas, both atomic and molecular, but no stars^{7,8}. All of these systems are generally more metal rich and more massive than the Magellanic bridge, but the observations by Muller *et al.*¹ show that the process of tidal galaxy formation seems to be efficient for

very small systems as well (even if the Magellanic bridge itself may be a bit too small to become a free galactic entity).

Tidally formed galaxies do not constitute a large population either in numbers or in mass and so will not have any great impact on the future evolution of our Universe. Their ultimate fate is probably to be swallowed by their parent galaxy, or any other large nearby galaxy. Despite their fleeting existence (from a cosmological perspective), they may still be very useful in studies of the structure of the large and extended haloes of their parent galaxies. How TDGs form, their dark-matter composition and their kinematics can tell us how the large parent galaxies themselves formed and how their dark-matter haloes are distributed. Although it is too early to draw conclusions about the CDM haloes from studies of these newly born tidal

dwarfs, continued observations may very well reveal something of this elusive dark matter that dominates our Universe. ■

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Medicine

Tackling multiple sclerosis

Hartmut Wekerle

New work in mice finds that certain anti-cholesterol drugs can reduce symptoms of disease in brain autoimmune disorders that are akin to human multiple sclerosis. There are also hints as to how the drugs might work.

Multiple sclerosis owes its enormous socioeconomic importance to several factors. Worldwide, as many as one million people are affected by the disease. It tends to afflict sufferers for most of their lives, often taking a severe, disabling course. And there are no effective treatments that stop multiple sclerosis in its tracks (although there are some that slow its progression). New therapies are desperately needed, and on page 78 of this issue Youssef and colleagues¹ investigate one attractive candidate — atorvastatin, a drug that is already used to reduce blood cholesterol levels in people with atherosclerosis or heart disease².

Multiple sclerosis is generally believed to develop when the body's immune cells — led by so-called helper T cells — attack myelin, the insulating, fatty sheath around nerve cells. This damages the myelin and the underlying neurons in both the brain and the spinal cord (Fig. 1, overleaf), leading to impaired transmission of nerve impulses and progressive physical disability. Treatments available today include one that involves engineered interferon- β proteins, which reduce the inflammation associated with nerve damage. Another is based on copaxone, a random composite of basic peptides, which probably activates brain-protein-detecting T cells that inhibit rather than support the autoimmune attack. Both drugs reduce the number of clinical relapses and the damage to the central nervous system (CNS). But both also come at a price —

quite literally in one sense, as they are very expensive. Moreover, they must be administered frequently by injection, which is a severe bother and carries a risk of side-effects. New treatments are needed that can be given orally and that, hopefully, also have a greater effect on the disease.

Enter Youssef *et al.*¹, who have looked at the effects of atorvastatin. This is a member of the statin group of molecules, which are commonly used to treat atherosclerosis and coronary disease². The authors find that the drug is effective against experimental autoimmune encephalomyelitis (EAE), an experimentally induced rodent autoimmune disease that is widely used as a model of human multiple sclerosis. By itself, this finding is perhaps not all that striking. After all, statins have already been used to reduce the rejection of human heart transplants by the immune system, and there have even been reports of a protective effect of injected statins in models of brain autoimmunity similar to EAE³. But what is particularly compelling about the new paper is that it provides a clue to the mechanisms by which statins might have anti-inflammatory effects.

Youssef *et al.* used three different models of mouse EAE. These differ in their genetics, the myelin-associated proteins targeted by the immune response, and the resulting 'clinical' diseases — they share basic features, but represent different phases of brain inflammation. After inducing EAE, the authors fed atorvastatin to the animals once a day for

several weeks, and analysed various parameters, such as the amount of CNS damage. They found that the treatment was beneficial in all three models. Moreover, it did not simply prevent the onset of EAE, but also — more importantly for a model of human autoimmunity — reduced established disease.

It seems that statins redirect myelin-specific helper T cells from the destructive role of causing disease to the beneficial task of suppressing autoimmunity. In particular, Youssef *et al.* found that the statins 'reprogramme' a subset of helper T cells (those that express the marker protein CD4). This reprogramming means that the cells no longer produce inflammatory cytokines — messenger molecules — such as interferon- γ and tumour-necrosis factor- β . Instead, they produce anti-inflammatory cytokines, including interleukins 4 and 10 and transforming growth factor- β . In agreement with this, key cytokine-inducing signalling pathways are also redirected in the statin-treated mice. Moreover, when the authors transferred CD4-expressing T cells from treated to untreated mice, those animals also were protected from EAE.

How do statins bring about such changes? These drugs are known to interfere with HMG-CoA reductase, an enzyme needed for cholesterol synthesis, and thus reduce the levels of blood cholesterol. Could such cholesterol reduction also affect the responses of helper T cells? This is a serious possibility, especially given that blood cholesterol modulates the antiviral responses of cytotoxic T cells⁴. But so far it is only known that cholesterol seems to reduce immune activity, so one might expect that statins, which lower cholesterol levels, would stimulate such activity — and that isn't the case in EAE.

The authors seem to favour another, not mutually exclusive, mechanism. They find that atorvastatin inhibits the expression by brain cells of a pivotal regulatory protein, CIITA, which steers the expression of MHC class II molecules. These molecules present peptides (antigens) to helper T cells for recognition. When the peptides are derived from invading microorganisms, the recognition sparks a useful immune response to these pathogens. But when class II molecules present antigens from the body's own cells, this stimulates a harmful autoimmune response such as that seen in multiple sclerosis and EAE.

In this proposed model, then, statins would work through CIITA and MHC class II molecules to decrease the presentation of 'self' antigens, thereby shifting the pattern of helper-T-cell activity. Previous work⁵ has shown that statin-mediated reduction in the expression of class II proteins is restricted to those of the body's cells that require a signal from interferon- γ to exhibit these proteins (which ties in nicely with Youssef *et al.*'s results). In contrast, the immune system's



Figure 1 Brain disease. This lithograph, produced by the nineteenth-century pathologist Jean Cruveilhier¹⁰, is one of the first illustrations of the brain and spinal-cord damage seen in multiple sclerosis. It shows the cerebellum (top) and spinal cord; the randomly distributed dark patches represent damaged areas.

'professional' antigen-presenting cells, such as dendritic cells, would be untouched. This is interesting, but raises questions. According to such a model, statin treatment would not affect dendritic cells in the peripheral immune system — but this is where they are primarily supposed to activate autoimmune T cells. It also remains unknown whether the

statin-induced reduction in CIITA expression in the CNS is caused directly by statins, or is an indirect consequence of reduced inflammation.

There is a third possible mechanism. Last year, Weitz-Schmidt *et al.*⁶ showed that statins partially block LFA-1, an adhesion molecule involved in T-cell migration and antigen presentation. LFA-1 is a component of the immune synapse — the well-organized site of contact between T cells and antigen-presenting cells. The molecule stabilizes this contact and thus controls the 'strength' of the antigenic signal⁷. Could partial interference with antigen presentation be involved in the T-cell redirection described by Youssef *et al.*? There are many examples in which the strength of antigen recognition by T cells determines the character of the effector molecules produced upon activation. But although this mechanism looks attractive, there are some questions. In particular, it remains to be seen whether EAE is affected by pravastatin, which does not block LFA-1, and by small-molecule LFA-1 blockers that do not inhibit HMG-CoA reductase.

Whatever the mechanism, the data reported by Youssef *et al.*¹ are intriguing, raising hopes of a new, oral treatment for multiple sclerosis and related diseases. But caution is warranted, because none of these three mouse models of EAE develops spontaneously; instead, they are induced by aggressive immunization protocols. So it is hard to use the results of testing potential drugs in these models to predict what will happen in human multiple sclerosis. For instance, interferon- γ aggravates multiple sclerosis, but reduces EAE. The opposite is true⁸ of the other main inflammatory cytokine, tumour-necrosis factor- α .

Also, the existing EAE models only represent pathogenesis mediated by CD4-expressing T cells. Emerging data suggest, however, that T cells expressing the other main marker protein, CD8, might also have a role in human multiple sclerosis⁹. This pathogenic pathway might not be affected by statins. But clinical trials of the effects of statins on human multiple sclerosis are now in progress, and could resolve these issues — hopefully in a favourable way.

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Suction feeding by a tiny predatory tadpole

This amphibian shoots its mouth forwards in a fish-like manner to suck in its prey.

Pipid tadpoles of the African genus *Hymenochirus* are not only among the smallest free-swimming, feeding vertebrates, but are also predatory suction feeders¹ — unlike other tadpoles, which typically ingest a suspension of organic particles. Here we use a high-speed video system to study details of the feeding mechanics of *Hymenochirus boettgeri* tadpoles and find that they first track individual prey organisms visually, and chase and then capture them by mouth using suction. This feeding mechanism is unique among frogs and is strikingly convergent with that used by teleost fishes.

Frogs of the genus *Hymenochirus* are unique in that both adults and tadpoles are predatory suction feeders^{1,2}. In most species of frog, adults use their jaws, tongue or forelimbs to capture prey², and tadpoles are usually suspension-feeding detritivores³ that use scraping mouthparts to create the suspension. This suspension is pumped into the mouth by rhythmic movements of the hyobranchial apparatus (throat skeleton)⁴; particles are trapped in the branchial basket, and water exits through the gill slits or a single spiracle^{3–5}.

Hymenochirus tadpoles, however, are morphologically divergent, lack a filter apparatus and scraping mouthparts, and have huge, frontally orientated eyes¹. Also, they are obligate air breathers and do not pulse-pump to irrigate the gills like most other tadpoles. Unaided visual observation indicates that they are predatory carnivores¹, but their minute size (the body length is less than 1 mm at first feeding) and rapid movements make the details of their feeding mechanics difficult to determine.

We used a high-speed video system (1,000 Hz) with a powerful macro lens that enabled us to follow the feeding behaviour and mechanics of *H. boettgeri* tadpoles (2–3 mm body length; Gosner stage 26). Our recordings reveal that they target each prey item visually, pursue it, then capture it by extension of a tubular mouth during an explosive buccal expansion (Fig. 1a, b; for movie, see supplementary information). Tadpoles complete their mouth extension within 2 ms and engulf the prey within 4 ms; prey travel into the mouth at 0.6 m s^{-1} . Buccal expansion is completed within 7 ms.

Comparably sized larval teleosts^{6,7} are slower feeders than these tadpoles, taking 4–12 ms to engulf their prey, which enter the mouth at $0.03\text{--}0.3 \text{ m s}^{-1}$. We calculated a Reynolds number of 300 for prey capture in tadpoles (from prey velocity and tadpole mouth diameter) compared with 5–70 for larval teleosts^{6,7}. The higher Reynolds number indicates that, although *Hymenochirus* is about the same size as larval teleosts, it is faster and better at overcoming the viscous drag that typically confronts small aquatic organisms⁸.

We compared our video images of moving *Hymenochirus* with preserved specimens and found that the tadpole's suction action is generated by a combination of hyobranchial movements (ceratohyal depression, basibranchial retraction) and cranial elevation; downward rotation of the lower jaw unfurls the soft tissues that comprise the extensible mouth (Fig. 1c).

After prey capture, the tadpoles expel water slowly (over 200 ms) through the paired gill slits of the reduced and simplified branchial basket¹ by raising the ceratohyals and basibranchial and lowering the head to their resting positions. The Reynolds number drops to 50 during water expulsion (Fig. 1a), and is lower for smaller *Hymenochirus* tadpoles when they begin feeding. Viscosity may thus be more problematic during water expulsion, as has been proposed for larval teleosts^{7,9}.

The feeding mechanism of *Hymenochirus* is remarkably like that of teleosts, which also suction-feed by using a combination of rapid mouth protrusion, hyobranchial depression and cranial elevation, followed by slower water expulsion through the gill slits^{6,7,9}. *Hymenochirus* and teleosts also share a hydrodynamically advantageous round mouth opening¹⁰.

Rapid mouth protrusion confers several benefits — it decreases the distance to the prey, accelerates flow through the mouth, restricts flow to the area in front of the mouth, and reduces the momentum (mass $\times$ velocity) that must be imparted to the water^{1,9}. Suction feeders that have non-protrusible mouths, such as some first-feeding larval fish⁷, must suck in more water than animals that have protrusible mouths. They therefore generate more momentum and suck themselves forwards^{6,9}. *Hymenochirus* imparts little momentum to the water, resulting in only a slight forward movement of the tadpole's body (Fig. 1a).

Miniaturized *Hymenochirus* tadpoles begin feeding at a smaller size than larval teleosts, which are universally suction

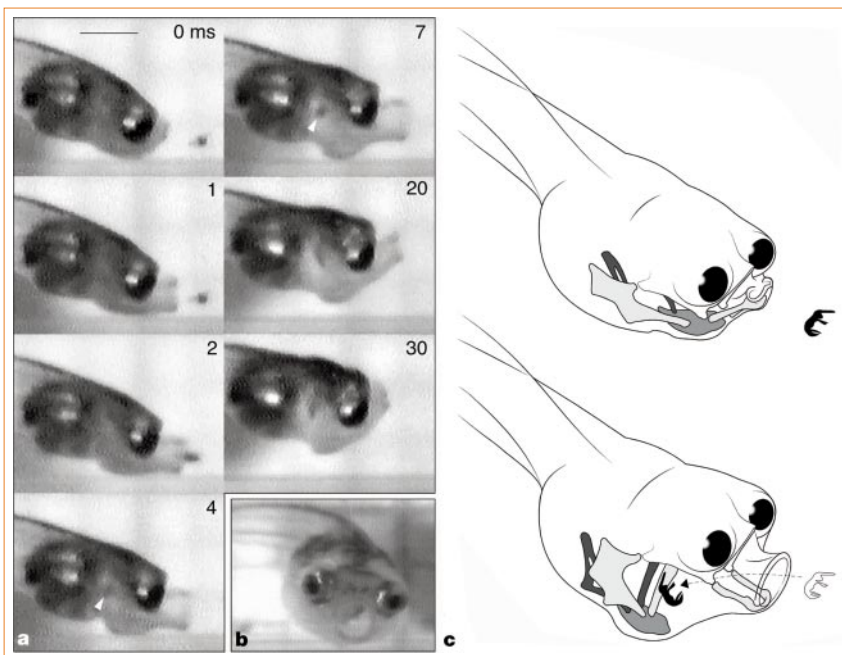


Figure 1 Morphology and function of suction feeding in the tadpole *Hymenochirus boettgeri* (body length, 2.6 mm). **a**, High-speed video sequence of feeding, showing hyobranchial depression, mouth extension and cranial elevation. Prey is engulfed within 4 ms of the commencement of mouth opening. White arrowhead in frame 4 indicates location of prey (the brine shrimp at the nauplius stage) in the pharynx. Scale bar, 1 mm. **b**, 'Yawning' tadpole, showing the hydrodynamically favourable round mouth aperture and the large, frontally orientated eyes. **c**, Dynamics of the tadpole's feeding apparatus, showing the mouth extension and hyobranchial movements that generate suction to ingest prey. Meckel's cartilage (lower jaw) and the ceratohyals are shown in light grey, the copula (basibranchial) is shown in medium grey, and the ceratobranchials are shown in dark grey.

feeders^{10,11}. What makes *Hymenochirus* so unusual is not just its size or feeding mode, but also that it is phylogenetically nested in a group of obligate suspension feeders^{3,12} and has independently evolved suction-feeding mechanics that are highly convergent with those of teleosts.

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Purinergic receptors

An ATP-gated ion channel at the cell nucleus

Transcriptional activity inside the nucleus of eukaryotic cells is regulated by ions such as calcium that need to be transported across the nuclear membrane. Here we show that an ion channel spanning the nuclear envelope between the cytoplasm and the nucleus could be regulated by an ATP-binding receptor of the P2X₇ subtype. Activation of this nuclear P2X₇ receptor by ATP in the cytoplasm may be a mechanism by which cellular activity can be coupled to changes in gene expression.

P2X₇ receptors are members of a family of ATP-binding receptors that are permeable to calcium. Originally thought to be absent from neurons¹, the P2X₇-receptor subunit P2X₇R has been shown to be targeted to excitatory but not inhibitory terminals, and yet it is absent from plasma membranes of the cell body^{2,3}.

To determine whether inhibitory neurons also express the receptor, we used *in situ* hybridization of the rat hippocampus to detect messenger RNA encoding P2X₇R. A positive signal was seen in the cytoplasm of all neurons in the cell-body layer³, 90% of which are excitatory cells⁴. In contrast to the localization of the P2X₇R protein to the terminals of only excitatory neurons, however, P2X₇R mRNA was also present in cells containing immunoreactivity for the potassium-channel subunit Kv3.1b, which identifies a subset of inhibitory neurons in the hippocampus⁵ (Fig. 1a, b).

The mismatch between the expression of P2X₇R mRNA and its protein in inhibitory neurons was explained when we used different antisera to stain the intra-

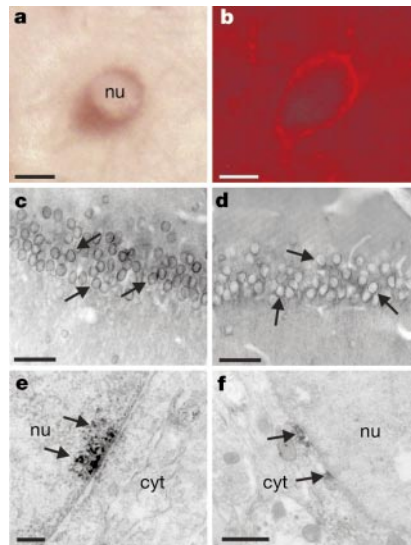


Figure 1 An ATP-gated ion channel spans the nuclear envelope. **a**, Messenger RNA encoding P2X₇R (visualized with alkaline phosphatase²) is present in the cytoplasm of all neurons in the hippocampus, and is shown in one neuron (nu, nucleus) adjacent to the cell-body layer; **b**, this neuron is identified as inhibitory by the presence of predominantly membrane-bound red Kv3.1b immunofluorescence (antibody against the K⁺-channel subunit Kv3.1b from Alomone Labs). **c**, **d**, Localization by light microscopy of P2X₇R protein: 'intracellular' (**c**) and 'extracellular' (**d**) epitopes are seen adjacent to the nuclear membrane (arrows) in all neurons in the cell-body layer. **e**, **f**, Immunoelectron microscopy of P2X₇R, seen here spanning the nuclear envelope, with **e**, its 'intracellular' portion adjacent to the nuclear side of the nuclear envelope (arrows), and **f**, its 'extracellular' portion facing the cytoplasm (cyt; arrows). Scale bars: **a**, **b**, 5 µm; **c**, **d**, 50 µm; **e**, **f**, 0.5 µm.

cellular (specific to amino-acid residues 576–595; 1:1,000 dilution; refs 2, 6) and extracellular (specific to amino acids 60–323; 1:100 dilution; ref. 6) portions of P2X₇R and found staining adjacent to the nuclear envelope in 100% of hippocampal

neurons (Fig. 1c, d).

The protein must span the nuclear envelope because the antisera against the two different epitopes labelled the cytoplasmic and inner surfaces of the nuclear membrane, respectively, with the ATP-binding site being in the 'extracellular' portion facing the cytoplasm (Fig. 1e, f). This finding still leaves unanswered the question of how P2X₇R is transported selectively to the presynaptic terminal in only excitatory neurons but to the nuclear envelope in all neurons.

Insertion of P2X₇R into the nuclear envelope is consistent with patch-clamp studies on nuclei showing that ATP binding maintains the open state of non-selective cation channels⁷ as well as inducing a macroscopic current⁸. The properties of P2X₇Rs correlate with these nuclear channels as they exhibit little or no desensitization⁹, they are activated by ATP (the 50% effective concentration is about 100 µM (ref. 9), which is well within the 5–10-mM range of cytoplasmic ATP), and they form a large pore upon prolonged activation⁹ (which may correspond to the ATP-induced macroscopic current in the nuclear membrane⁸ and the increase in envelope permeability induced by ATP binding⁷).

Moreover, the ATP-gated nuclear channels^{7,8} and the P2X₇R⁹ are both permeable to Ca²⁺ ions, and changes in nuclear Ca²⁺ concentration are linked to changes in the transcription of several genes implicated in neuronal plasticity (principally by regulating the activity of the transcriptional co-activator protein CBP)¹⁰. The presence of P2X₇R in the nuclear envelope of phenotypically heterogeneous neurons and of ATP-gated channels in nuclei of diverse cell types (such as *Xenopus* oocytes and mouse liver cells) therefore has wide implications as it provides a means of regulating nuclear Ca²⁺ concentration in response to cytoplasmic activity.

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Structure of a T7 RNA polymerase elongation complex at 2.9 Å resolution

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The single-subunit bacteriophage T7 RNA polymerase carries out the transcription cycle in an identical manner to that of bacterial and eukaryotic multisubunit enzymes. Here we report the crystal structure of a T7 RNA polymerase elongation complex, which shows that incorporation of an 8-base-pair RNA–DNA hybrid into the active site of the enzyme induces a marked rearrangement of the amino-terminal domain. This rearrangement involves alternative folding of about 130 residues and a marked reorientation (about 130° rotation) of a stable core subdomain, resulting in a structure that provides elements required for stable transcription elongation. A wide opening on the enzyme surface that is probably an RNA exit pathway is formed, and the RNA–DNA hybrid is completely buried in a newly formed, deep protein cavity. Binding of 10 base pairs of downstream DNA is stabilized mostly by long-distance electrostatic interactions. The structure implies plausible mechanisms for the various phases of the transcription cycle, and reveals important structural similarities with the multisubunit RNA polymerases.

Gene expression in all organisms requires messenger RNA synthesis by DNA-dependent RNA polymerase (RNAP). These enzymes can be divided into two classes: multisubunit (bacteria, archaea, eukaryotes) and single-subunit (some bacteriophages, mitochondria, chloroplasts) RNAPs. Although they share no apparent sequence or structural homology, the RNAPs of both classes carry out the basic steps of transcription in an identical manner¹. To initiate RNA synthesis, the enzyme must bind to a specific promoter DNA sequence that lies upstream of the start site for transcription, separate (melt) the two strands of the DNA in the vicinity of the start site (forming a transcription bubble), and begin RNA synthesis using the coding strand of the downstream DNA as a template and a single ribonucleotide as a primer. During the early stages of transcription, contacts between the RNAP and the upstream promoter sequences are maintained while the active site translocates downstream, resulting in the formation of a short RNA–DNA hybrid and extension of the transcription bubble^{2–5}. The T7 RNAP initiation complex (IC) is unstable and repeatedly releases short (3–8 nucleotides) abortive RNA products before it undergoes a transition to form a stable elongation complex (EC)⁶. The transition starts when the RNA–DNA hybrid reaches a length of 8–9 base pairs (bp) and results in promoter release, collapse of the melted promoter region, and displacement of the 5' end of the nascent RNA^{4,5,7}. During elongation the length of the RNA–DNA hybrid is maintained at 7–8 bp and the transcription bubble closes just after the RNA chain peels away from the DNA template^{8–10}.

The solution of several RNAP structures has resulted in a breakthrough in our understanding of the functional aspects of transcription^{11–20}. The structures of T7 RNAP–nucleic acid complexes revealed that promoter binding involves three principal structural motifs of the RNAP: an (A + T)-rich recognition loop interacts with the –17 region; a specificity loop makes base-specific contacts around –9; and an intercalation loop involving Val 237 facilitates promoter melting and stabilizes the upstream edge of the separated transcription bubble between –5 and –4 (the start site for transcription is designated as +1). The structure of an early IC in which the first three bases of the template strand have been transcribed is

essentially unchanged from that of the binary promoter complex, indicating that the initial stages of transcription may be achieved without major changes in enzyme structure. However, the structure of the IC did not allow space in the active site for an RNA–DNA hybrid greater than 3 bp, and therefore did not provide a plausible mechanism for further transcription progress. The crystal structure of the T7 RNAP elongation complex reported here reveals that incorporation of an 8-bp-long RNA–DNA hybrid into the active site results in unprecedented structural alterations of protein structure, thereby resolving the principal differences between previous biochemical and structural data^{9,14,21}. Although the conformation of the carboxy-terminal portion of T7 RNAP (which includes the active site) resembles that of polymerase I-like DNA polymerases^{11,22,23}, the overall organization of the EC is remarkably similar to that of the multisubunit RNAPs, reflecting the common functions of these two classes of RNAPs.

Structure determination and overall structure

Elongation complexes were formed and crystallized as described^{24,25}. The structure was refined at 2.9 Å resolution to a final $R = 23.5\%$ and $R_{\text{free}} = 28.4\%$ (Fig. 1a, b; see also Supplementary Table 1).

The RNAP in the EC has a shell-like, highly porous architecture (Fig. 1c–e). The downstream DNA is bound in a deep groove and enters through a wide passage to a cavity that contains an 8-bp RNA–DNA hybrid. The axis of the hybrid is nearly perpendicular to the entering DNA, as in the yeast RNA polymerase II elongation complex¹⁷. The structure contains partly accessible channels, which presumably correspond to binding sites for the template and non-template DNA strands, and prominent pores for entry of the substrate and exit of the RNA product. The positive charge covering almost the entire interior of the molecule extends through the pores and channels to the external surface. The channels and pores are features characteristic only to the EC.

Protein structure

During the formation of the EC, the N-terminal domain of the

RNAP (residues 2–266) undergoes remarkable structural reorganization as compared with the IC (Fig. 2). This involves a marked reorientation of a core subdomain (72–151, 206–257), which remains unaltered, and an alternative folding of approximately 130 residues. These changes result in the formation of three structural elements: an N-terminal extension (N-subdomain; residues 2–71), a central flap-like subdomain (152–205), and a C-terminal linker that connects the N-terminal domain to the C-terminal portion of the enzyme (C-linker; 258–266) (Fig. 2).

Relative to the IC (Protein Data Bank accession number 1QLN¹⁴; Fig. 2b, c), the core subdomain does not change its internal structure (root mean square deviation, r.m.s.d. = 0.7 Å over 524 main chain atoms) but moves as a rigid body (35 Å translation and 130° rotation) to its final position in the EC (Fig. 2a, c). This reorientation opens space in the active site to accommodate the expanding RNA–DNA hybrid. Of note, the core exhibits high intrinsic structural similarity (but lacks sequence homology) to a C-terminal region of T7 RNAP and to a lesser extent to the corresponding segment of pol I-like DNA polymerases (Taq DNAP; Protein Data Bank accession number 1TAQ²⁶) (Fig. 3). This suggests that the core subdomain evolved from duplication of a conserved structural element.

In the IC (Fig. 2b, c), the N-subdomain does not interact with nucleic acids, and its fold consists of seven structural motifs: loop-helix-loop-helix-loop-helix-disordered region. In the EC (Fig. 2a,

c), the N-subdomain adopts a more compact loop-helix-loop-helix-loop conformation that constitutes a portion of the binding site for the RNA–DNA hybrid. Despite the marked difference in folding, the N-subdomains in the IC and EC are located on the same side of the RNAP molecule, so that a short α -helical fragment (31–43) coincides in both structures. This suggests that the N-subdomain keeps a fixed orientation during the structural transition of the RNAP, and that all alterations in this domain result from alternative folding.

A long, unfolded loop (165–205) intervenes between the N- and C-terminal portions of the core in the IC (Fig. 2b, c). In the EC (Fig. 2a, c) this loop is folded into the flap subdomain, which consists of a helix-turn-helix (HTH) motif followed by a C-terminal loop. In the EC, the flap subdomain spans the interval between the upstream end of the RNA–DNA hybrid and downstream DNA.

Finally, whereas the short C-linker connecting the N-terminal domain to the C-terminal domain has a random coil conformation in the IC (Fig. 2b, c), it adopts an α -helical conformation in the EC (Fig. 2a, c) and extends the C-terminal α -helix of the core subdomain towards the end point of the N-terminal domain.

The C-terminal, DNA polymerase-like portion of the RNAP, which consists of three major domains designated as ‘fingers’, ‘palm’ and ‘thumb’^{11,12}, remains largely unaltered in the EC as compared with the IC (Fig. 2a, b). The primary exception is the specificity loop (739–772)^{13,27,28} (Fig. 2a, b). A substantial shortening of the loop hairpin (by about 13 Å) is probably the result

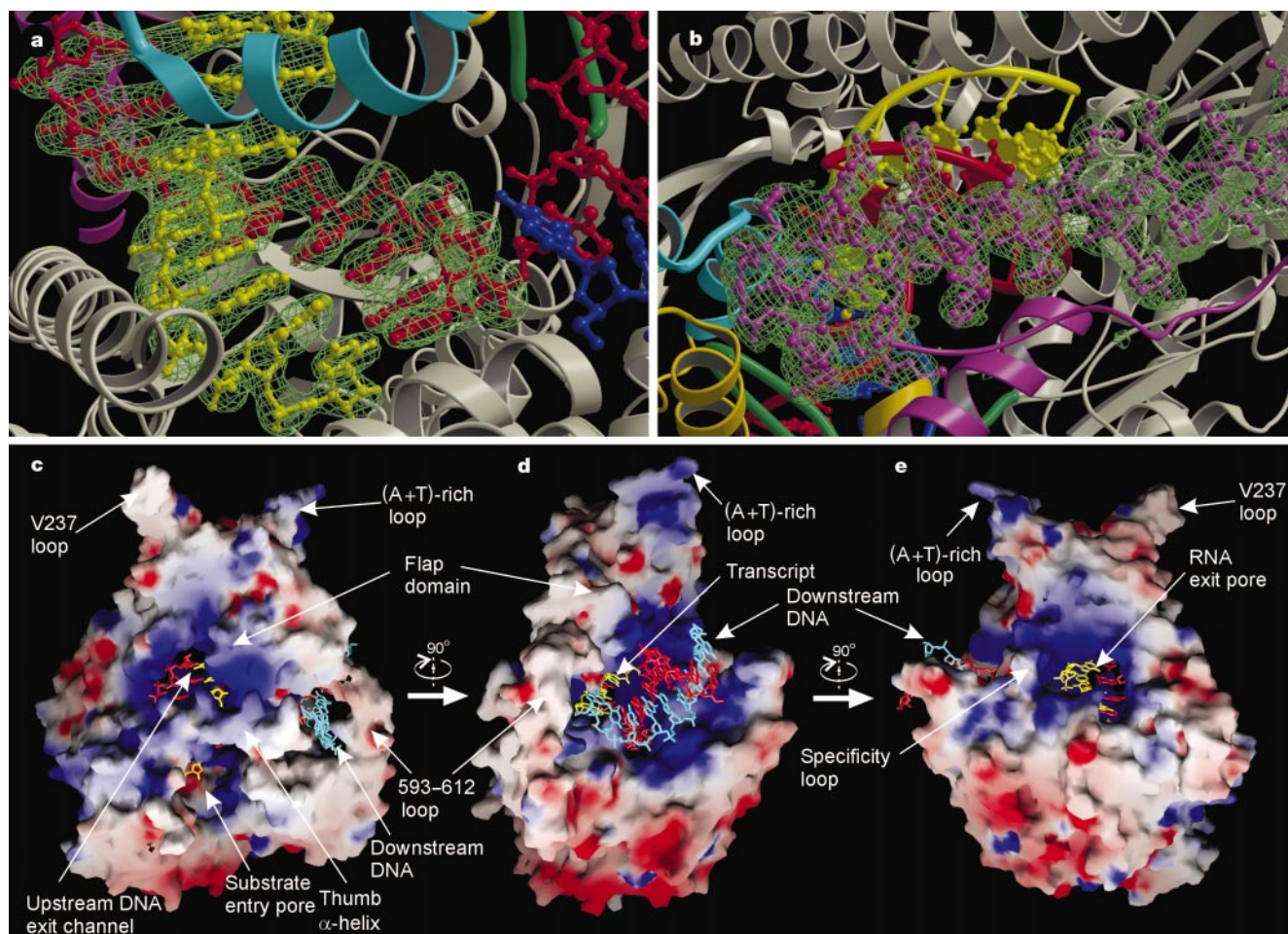


Figure 1 The T7 RNAP EC crystal structure. The RNA (light yellow), DNA template (red) and non-template (blue) strands are shown as a ‘ball-and-stick’ model. **a**, **b**, The $|F_o - F_c|$ omit electron density map (3.2 σ contour level; green) produced for the RNA–DNA hybrid (**a**) and for the long N-terminal α -helix (residues 31–62, ball-and-stick model; magenta)

(**b**) is superimposed on the atomic model. The rest of the protein is represented by a ribbon diagram. **c–e**, Three views of the EC. The protein surface is coloured according to the electrostatic potentials (positive, negative and neutral are dark blue, red and white, respectively).



subdomain and with residues in the first turn of the flap subdomain. The DNA strands are separated just before the template strand enters the active site by interaction with the N-terminal part of a helix in the fingers subdomain (642–662), which we designate a ‘downstream DNA zipper’. DNA melting involves stacking of the template base at +2 on the Phe 644 side chain of the zipper, which appears to cause the base at +1 to flip out, thereby directing the template strand towards the active site (Fig. 4b). (Nucleotide positions in the EC are designated relative to the active site at +1.) The first unpaired nucleotide of the non-template strand (at +1) is directed towards a deep groove formed by a loop (593–610) and a helix-turn-helix fragment (647–675) from the fingers subdomain, part of the flap subdomain, and the N-terminal portion of the long α -helix from the thumb subdomain (Fig. 1c, d). This groove extends to the template strand exit located between the second turn of the flap subdomain and the C-terminus of the long helix of the N-terminal extension (Fig. 1c).

The 8-bp RNA–DNA hybrid in the EC adopts an underwound A-form conformation of the double helix that is similar to the conformation of the RNA–DNA hybrid in the yeast RNA polymerase II EC (Fig. 1a; r.m.s.d. 0.6 Å)¹⁷. The hybrid-binding cavity complements this shape and has a surface that consists of alternating hydrophobic and positively charged residues, which may facilitate translocation (Fig. 4a). The first 3 bp at the downstream end of the hybrid interact with the protein in a similar

fashion as in the T7 IC (Fig. 4a). However, the interactions of the 3 bp at the upstream end of the hybrid involve newly folded elements of the EC. Most notable are interactions of template strand nucleotides at positions –6, –7 and –8 with the C-terminal portion (50–60) of the long helix from the N-subdomain, and the RNA nucleotides at positions –6 and –7 with the flap subdomain (Fig. 4a).

The upstream boundary of the RNA–DNA hybrid cavity is defined by the C-terminal part of a loop-helix motif (62–71) that connects the long helix of the N-subdomain with the core subdomain. The Gln 58, Glu 63 and Asp 66 side chains in this loop project towards the plane of the base pair at the upstream end of the hybrid at a distance of about 7.3 Å (Fig. 4a, c), which may allow hybrid extension by 1 bp without serious protein alterations.

RNA exit and substrate entry pores

In the EC, a highly positive pore leads from the upstream end of the RNA–DNA hybrid to the surface, and probably provides an RNA exit pathway^{29,30} (Fig. 1e). The pore is formed by part of the reorganized specificity loop, the newly formed C-linker, and part of the C-terminal portion of the enzyme (291–303). A final step in the transition from the IC to EC occurs when 10–14 nucleotides of RNA have been synthesized^{31,32}, and may result from interactions of the displaced RNA with the surface of the pore, thereby stabilizing the transcription bubble.

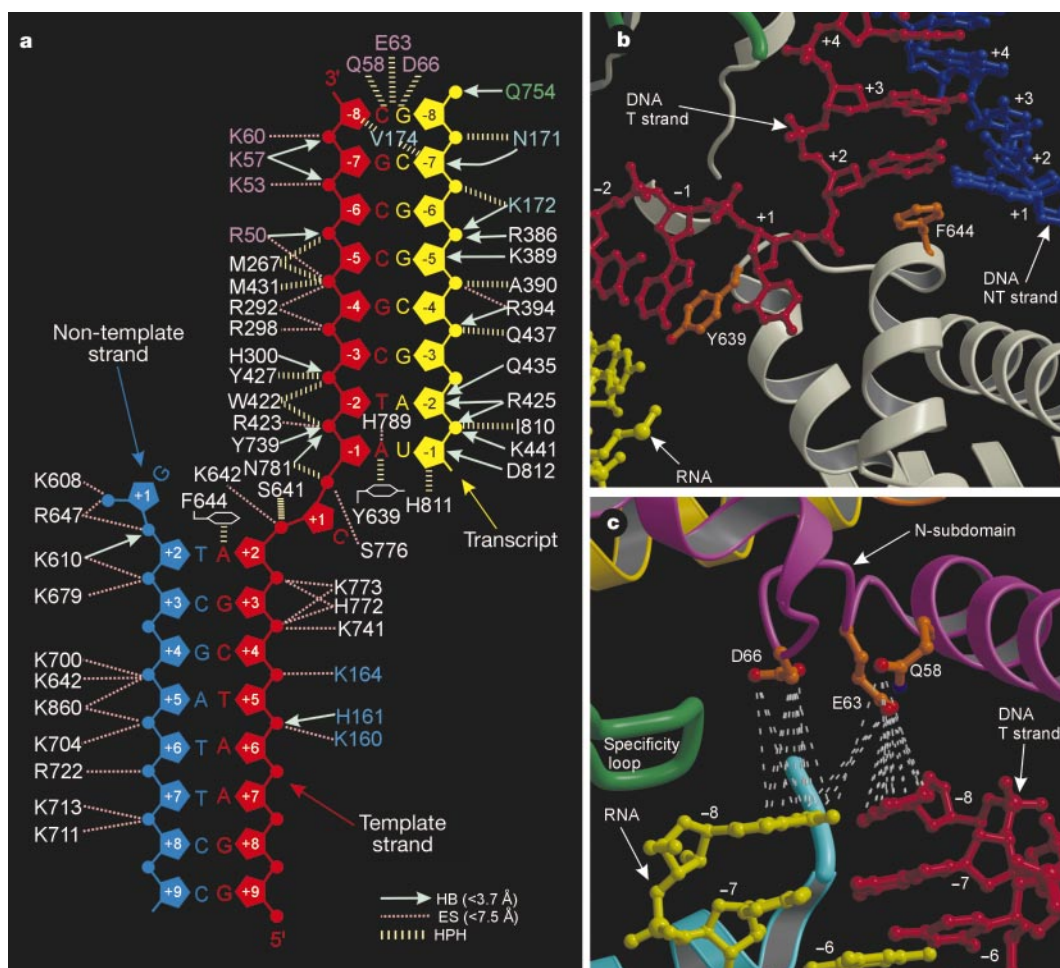


Figure 4 Protein–nucleic acid interactions. **a**, Schematic drawing of the protein–nucleic acid interface. HB, hydrogen bonds; ES, electrostatic interactions; HPH, hydrophobic interactions and closely approaching residues with the nucleotide indicated (including phosphate and sugar). **b**, Junction between downstream DNA and the RNA–DNA hybrid.

c, Upstream end of the RNA–DNA hybrid. Colours are the same as in Fig. 2. The side chains of Tyr 639 and Phe 644 (**b**), and Gln 58, Glu 63 and Asp 66 (**c**) are shown in orange. Proximity of the N-subdomain residues to the upstream hybrid base pair (**c**) are indicated by dashed lines. T, template; NT, non-template.

Another pore provides access to the active site, and is probably the pathway for entering substrate (Fig. 1c). Although this opening is also present in the IC, it becomes more accessible in the EC as a

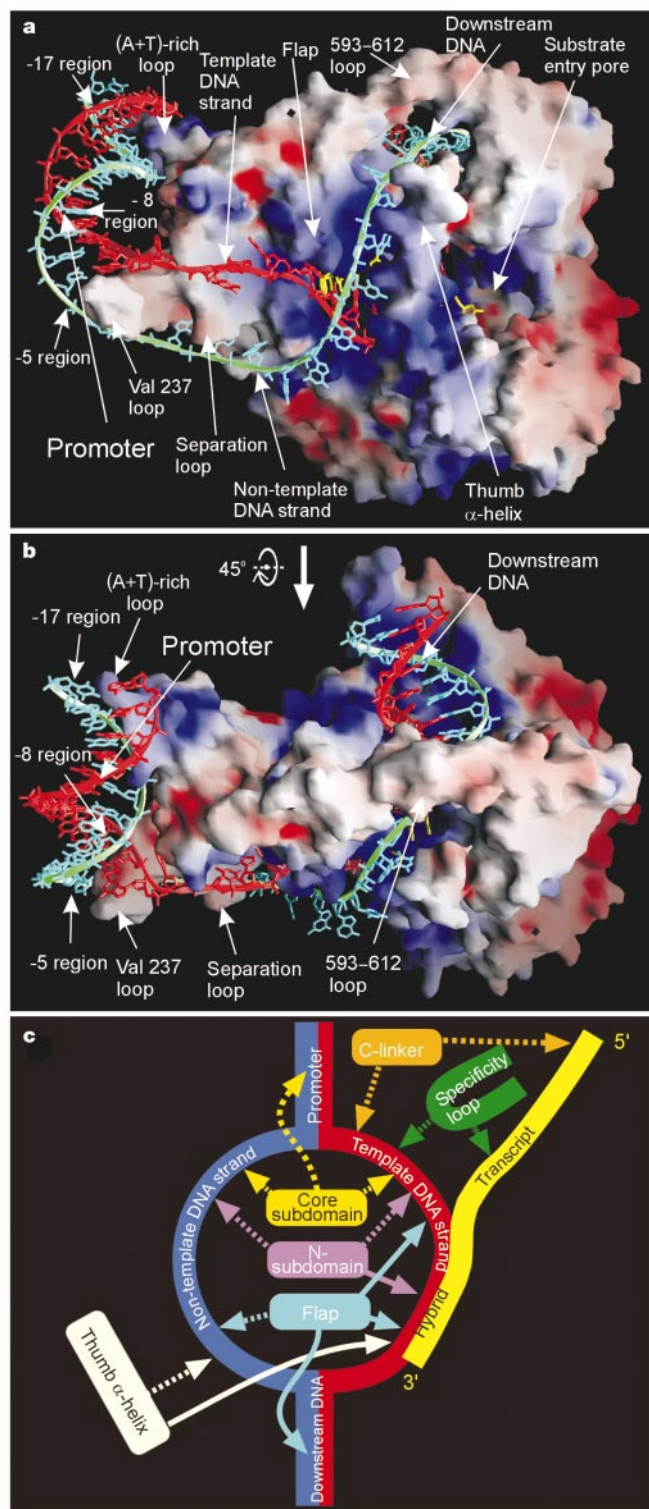


Figure 5 Model of the late IC. Two different views are shown in **a** and **b**. The protein is represented by the same electrostatic potential surface as in Fig. 1c–e. The nucleic acids are shown as a combination of ribbon phosphate backbone and ball-and-stick models with the RNA, and DNA template and non-template strands coloured in light yellow, red and light blue, respectively. **c**, Interaction of the protein domains that have principal roles in the structural organization of the late IC (dashed arrows) or the EC (solid arrows), with the components of the transcription bubble shown schematically. The colour scheme is the same as in Fig. 2.

result of a 12° bend of the thumb domain α -helix (372–409) (Fig. 1d). Substitutions of residues that line the surface of the pore result in marked effects on catalytic activity^{33–36}, which would be consistent with a role for this region in providing a path for entering substrate, or, owing to its proximity to the active site, on catalysis.

The presence of these pores in the EC provides a clear parallel to the multisubunit RNAPs. However, whereas these exit and entry pathways are short in the T7 EC, they are considerably longer (about 30 Å) in the multisubunit RNAPs^{15,16}. This difference may account, in part, for the high rate of polymerization exhibited by T7 RNAP (200 nucleotides per s for T7 compared with 30 nucleotides per s for *Escherichia coli* RNAP)¹.

The transition from an IC to an EC

Biochemical experiments have shown that during the late stages of initiation, just before promoter release, the length of the RNA–DNA hybrid is about 8 bp, as is observed in the EC⁷. It therefore seems likely that the conformation of the RNAP in the late IC resembles that of the EC. A number of questions arise concerning the transition from an early IC to a late IC. Does the transition occur in one step, or is it a multistep process? Furthermore, how are the upstream promoter contacts maintained while the hybrid grows to 8 bp?

Modelling of the RNA–DNA hybrid as it is extended from 3 bp in the early IC indicates that the first clash of the hybrid would occur with elements of the core domain at 4 bp, but that rotation of the core domain, together with specificity loop and promoter, could accommodate extension of the hybrid to 5 bp without substantial refolding (consistent with biochemical data^{2,37}). After 5 bp, however, the structure of the EC shows that the hybrid is greatly stabilized by interactions with the refolded elements of the N-terminal domain (Fig. 4a), suggesting that reorganization starts at this point. This is consistent with the observation that mutations affecting the elements in the core domain that interact with the hybrid at this length inhibit extension beyond 5 nucleotides^{38,39}. The (A + T)-rich recognition and the Val 237 intercalation loops are part of the unaltered core subdomain, and the promoter contacts mediated by these elements may be retained during the reorientation of this subdomain (assuming that the upstream DNA moves along with the core) and might persist up to 8–9 bp. On the other hand, modelling suggests that this reorganization cannot occur without loss of contacts between the specificity loop and the promoter (Fig. 2a). This is consistent with the results of ultraviolet laser crosslinking experiments, which show that promoter contacts at –7 and –8 are lost before the contact at –17 (ref. 5).

The pattern described above suggests that a simple superposition of the IC and EC core subdomains could provide a plausible model for continued binding of the upstream promoter region in the late IC (Fig. 5). The model shows that a transcription bubble of 9–10 nucleotides (which corresponds to a hybrid length of 5–6 bp) would be too short to connect the promoter in the late IC with the hybrid and downstream DNA (not shown). Thus, the rearrangement that is proposed to commence at 6 bp cannot occur as a single step, and is probably a multistep process in which gradual reorganization of the N-terminal domain is accompanied by incremental movement of its core. The 13–14 nucleotide length of the bubble in the late IC (which corresponds to a hybrid of 8–9 bp, and is in agreement with recent fluorescence experiments⁷) suggests a probable pathway for the template and non-template strands that connect the upstream region of the promoter with the hybrid and downstream DNA (Fig. 5). A 25 Å-long positively charged groove, formed by the thumb domain α -helix and the flap subdomain, is likely to form a binding site for approximately 6–8 nucleotides of the downstream non-template DNA strand^{29,30} (Fig. 5). At the upstream edge of the transcription bubble there are two hydrophobic cavities that can accommodate 4 bases of template and 4–5 bases of non-template strand DNA (Fig. 5). The central wall separating these two cavities

(the separation loop) is formed by a portion of the core domain (128–133), whereas the remaining portions are formed by newly formed structural elements. If the movement of the core domain is fixed at this point, further extension of the RNA–DNA hybrid would result in promoter release as a consequence of the strain associated with extension, and/or as a result of compression and extrusion of the template and non-template strands from the hydrophobic channels. Biochemical data suggest that promoter release may precede the final collapse of the transcription bubble and displacement of the 5' end of the RNA from the hybrid⁷. Interaction of the separation loop with the template and non-template strands just downstream of the promoter may prevent complete collapse of the bubble until later in the initiation process. In the model, the non-template base at –11, which would later re-anneal to the template strand to form the upstream edge of the bubble after promoter release, is on the protein surface in close proximity to the upstream edge of the RNA–DNA hybrid. This suggests that the final transition to the EC may occur without further major alterations of protein structure.

Structural organization of the EC

The EC structure, in combination with modelled promoter DNA and a plausible trace of the template and non-template strands, comprises a transcription intermediate that corresponds to the late IC. Although the modelled nucleic acids are not suitable for detailed analysis of the interactions, they allow a discussion of the role and significance of new structural elements that are acquired by the RNAP in the course of refolding.

The EC structure shows that the rearranged segments of the N-terminal domain (N- and flap-subdomains, and C-linker) are principal elements of the complex. These motifs, which do not have an essential functional role in the early IC, become multi-functional in the late IC and in the EC, and are extensively involved in interactions with various components of the transcription bubble (Fig. 5c).

The N terminus of the N-subdomain probably provides a pathway for the template and non-template DNA strands in both the late IC and the EC. The long α -helix of the N-subdomain interacts with the RNA–DNA hybrid in the EC and may also contribute to the formation of the upstream template and non-template DNA-binding sites in the late IC (Fig. 5c). Importantly, the C-terminal linker region of the N-subdomain is in close proximity to the upstream boundary of the hybrid in the EC, and extension of the hybrid by 1 bp would clash with the hydrophilic residues clustered at the tip of this loop (Fig. 4a, c). The loop may therefore act as a 'hybrid zipper' to facilitate strand separation of the hybrid. In bacterial RNAPs, the σ -subunit 'destabilizer' loop, which is also likely to interact with the upstream edge of the hybrid, contains two highly conserved acidic residues at the tip that may have essentially the same role¹⁹.

In the EC structure, the flap subdomain interacts both with the upstream end of the hybrid and with the downstream DNA. In the late IC, it is probably involved in binding of the upstream portion of the template strand. The flap subdomain also forms part of a potential non-template binding site in both the late IC and the EC (Fig. 5c). Finally, in the EC, this subdomain is required to fix the position of the specificity loop (Fig. 2a), which is indispensable for the formation of the RNA exit channel. Thus, the flap subdomain seems to be of central importance to the integrity and function of the late IC and the EC. Consistent with this, mutations within this motif result in a failure to resolve the transcription bubble^{31,40–42}.

Although very short (8 residues), the C-linker also probably interacts with two distinct elements of the transcription bubble: with the upstream DNA template strand in the late IC and with the displaced RNA product in the RNA exit pore (Fig. 5c).

Other than the N-terminal domain, the principal element whose

functional purpose is switched during the transition to an EC is the specificity loop. While losing its role in promoter recognition it forms a portion of the RNA exit channel and is probably involved in interactions with the displaced RNA product. The flexible out-looped segment of the loop, which is rich in hydrophilic residues, may intrude into the RNA exit pore. We speculate that this segment may be important for responding to pausing and termination signals. The tip of the specificity loop may also interact with the DNA template strand in the late IC (Fig. 5c).

Abortive cycling

Four structures of T7 RNA have been determined previously: free enzyme; RNAP bound to the transcription inhibitor T7 lysozyme; a binary RNAP–promoter complex; and an early IC^{11–14}. All of these structures display very similar conformation of the enzyme (Fig. 2b), which implies that this conformation probably represents the most stable organization of the protein. In contrast, the conformation of RNAP in the EC (Fig. 2a) has never been observed before. The extensive protein–nucleic acid interface (about 2,500 Å²) in the EC, which is lacking in the other structures, suggests that the protein conformation of the EC is greatly stabilized by these interactions. During the transition to an EC the exposed hydrophobic surface of the N-terminal domain increases by roughly 1,400 Å², which, in the absence of the nucleic acid–protein interface, may favour a return to the more stable IC conformation. Given the probable multistep nature of the transition from the early IC to the late IC, it seems probable that the intermediate enzyme conformations would be less stable than the one observed in the EC, because they lack the full extent of the final nucleic acid interface. In addition, modelling shows that the principal N-subdomain and C-linker α -helices can only be gradually folded during the course of the transition, and that the unfolded IC loop, comprising the flap domain in the EC, would lose its stabilizing interactions with the protein during the initial stage of the transformation. We speculate that competition between the nascent RNA–DNA hybrid for space in the active site (which induces the reorganization of the RNAP) against the tendency of the partially folded protein structures to fold back to the initial conformation is a major factor contributing to abortive cycling. A similar mechanism has been proposed for bacterial multisubunit enzymes, in which the growing RNA–DNA hybrid competes with initiation factor- σ resulting either in abortive RNA synthesis or displacement of σ to form a stable EC^{18,19}.

Discussion

On the basis of footprinting experiments, two principal models were proposed for the progress of transcription initiation: 'polymerase inchworming', in which the RNAP structure extends to include the expanding transcription bubble, and 'DNA scrunching', in which the RNAP structure remains unaltered while the bubble is accumulated in the active site. The interpretation of previous structural data favoured the scrunching model, in which the RNA–DNA hybrid length was limited to 3 bp and the strain associated with packing of the transcription bubble in the unaltered enzyme led to abortive initiation¹⁴.

Although not excluding a role for scrunching during the early stages of transcription (when the hybrid is ≤ 4 bp), the T7 RNAP EC structure reported here provides strong evidence in favour of polymerase inchworming during the later stages of initiation, as the structure of the RNAP is expected to change progressively to accommodate the RNA–DNA hybrid as it grows to a final length of about 8–9 bp.

During the maturation of the EC, the elaboration of the components of the transcription bubble both induce and stabilize the reorganization of the protein structure. We suggest that a similar role for nucleic acids may also be expected for other multifunctional nucleic-acid-binding proteins, including the multisubunit RNAPs during their transition from an IC to an EC.

The overall organization of the T7 RNAP EC bears a marked resemblance to that of the multisubunit RNAPs. Moreover, the structural mechanism by which T7 RNAP achieves this final state is similar to the steps that are observed in bacterial RNAPs. In both cases the active site that accommodates the components of the mature transcription bubble is greatly reduced at the beginning stages of transcription, and the RNA exit channel is either not formed or is blocked. These elements probably form gradually in a process that involves competition between the growing RNA–DNA hybrid and protein structural elements. The reorganization observed in T7 RNAP therefore resembles the displacement and release of the bacterial σ -factor, and we suggest that a similar situation may exist in the multisubunit eukaryotic RNAPs. The induced reorganization of the enzyme as it matures and makes the transition to an EC is therefore probably a consistent theme among DNA-dependent RNAPs. □

Methods

Crystallization and data collection

Stable, transcriptionally competent complexes were formed by mixing RNAP at a 1:1 molar ratio with a nucleic acid scaffold consisting of an 18-nucleotide template DNA strand annealed to 10 nucleotides of downstream DNA and a 12-nucleotide RNA primer, of which 4 nucleotides at the 5' end were unpaired with the template. Such complexes exhibit all of the properties of a promoter-initiated EC²⁴.

Crystallization was carried out by the sitting-drop vapour diffusion technique at 20 °C (ref. 25). The drop, containing 2 μ l of 10 mg ml⁻¹ complex solution, was mixed with 2 μ l of well solution containing 10% PEG 8000, 10% glycerol, 5 mM β -mercaptoethanol, 100 mM Tris buffer, pH 8.1. Thin, plate-like crystals (0.5 $\times$ 0.5 $\times$ 0.05 mm) were grown within 3–5 days and diffracted to 2.6 Å resolution using synchrotron X-ray radiation. The T7 RNAP EC crystals belong to the *P1* space group with unit cell dimensions $a = 79.91$, $b = 84.97$, $c = 202$ Å, $\alpha = 90.36$, $\beta = 92.97$, $\gamma = 109.94^\circ$. A complete diffraction data set was collected at 100 K on SPRING-8 beam line BL45XU, and processed with the HKL2000 program package⁴³ (Supplementary Table 1).

Structure determination and refinement

The structure was determined by molecular replacement using the coordinates of the T7 RNAP IC (Protein Data Bank accession number 1QLN¹⁴) as a search model⁴⁴. Clear molecular replacement solutions for four crystallographically independent molecules were obtained only after removal of the N-terminal domain (266 residues) and all protruding segments of the C-terminal domain from the initial model, which indicated large structural alterations of the omitted portions of the molecule²⁵. The phases obtained from the initial structure were improved by density modification and non-crystallographic symmetry (NCS) averaging using the DM program⁴⁴, resulting in an interpretable electron density for the omitted N-terminal domain and the RNA–DNA hybrid. The atomic model was built on the basis of this density using the O program⁴⁵ and then refined by the CNS program⁴⁶. At this stage, however, the refinement was unstable, resulting in large portions of density that disappeared from the ($2F_o - F_c$) electron density maps after additional refinement cycles. Careful inspection of diffraction data revealed barely separated twin spots at medium resolution, which become more profound at high resolution. This was characteristic of all crystals examined. To avoid this problem, the size of integration spots was increased at the stage of data processing to include both portions of the spots twinned at high resolution, and the diffraction intensities were calculated using summation rather than profile fitting to allow for better evaluation of the intensities for the two peaks in the integrated area. The four-fold NCS averaging appeared to be a powerful tool for the evaluation of the quality of the diffraction data (see Supplementary Information section 'NCS averaging'). The reprocessed data at 2.9 Å resolution resulted in clear electron density, which allowed for unambiguous modelling of the missing regions of the molecules with almost all protein side chains. The four independent molecules in the asymmetric unit are almost identical, with r.m.s.d. between the molecules ranging from 0.77 to 1.12 Å for the protein main chain and nucleic acid atoms. The downstream DNA is flexible in two molecules, and two regions of the protein were not resolved (757–765 and 352–371). Only weak electron density was visible for the four unpaired nucleotides (AACU) in the 5' region of the RNA and for the last base pair of the downstream DNA (G•C, position +10). Figures 1a, b, 2a, b, 3 and 4b, c, were prepared using the programs Molscript⁴⁷, Bobscript⁴⁸ and Raster3D⁴⁹. Figures 1c–e and 5 were prepared using the GRASP program⁵⁰.

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Gravitationally redshifted absorption lines in the X-ray burst spectra of a neutron star

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The fundamental properties of neutron stars provide a direct test of the equation of state of cold nuclear matter, a relationship between pressure and density that is determined by the physics of the strong interactions between the particles that constitute the star. The most straightforward method of determining these properties is by measuring the gravitational redshift of spectral lines produced in the neutron star photosphere¹. The equation of state implies a mass–radius relation, while a measurement of the gravitational redshift at the surface of a neutron star provides a direct constraint on the mass-to-radius ratio. Here we report the discovery of significant absorption lines in the spectra of 28 bursts of the low-mass X-ray binary EXO0748–676. We identify the most significant features with the Fe XXVI and XXV $n = 2$ –3 and O VIII $n = 1$ –2 transitions, all with a redshift of $z = 0.35$, identical within small uncertainties for the respective transitions. For an astrophysically plausible range of masses ($M \approx 1.3$ –2.0 solar masses; refs 2–5), this value is completely consistent with models of neutron stars composed of normal nuclear matter, while it excludes some models^{6,7} in which the neutron stars are made of more exotic matter.

The XMM-Newton observatory⁸ observed the low-mass X-ray binary EXO0748–676 (ref. 9) during its commissioning and calibration phases for almost a half million seconds, spread over six satellite orbits between 21 February 2000 and 21 April 2000. Data were recorded with the Reflection Grating Spectrometer¹⁰ (RGS) for 335,000 s (data obtained with the European Photon Imaging Cameras^{11,12} (EPIC) are available for 39,000 s of simultaneous exposure). During this time, a total of 28 X-ray bursts were recorded with the RGS, lasting a cumulative 3,200 s. During the brief bursts, the neutron star outshines the accretion-generated light by an order of magnitude in intensity, while the continuing accretion ensures a continuing supply of heavy elements in the stellar photosphere. This makes the burst spectrum a promising place to detect absorption structure from a neutron star photosphere, a long-standing goal in compact-object astrophysics. With a detailed stellar photospheric spectrum, the techniques of classical stellar spectroscopy could be used to measure the fundamental parameters of neutron stars. It should also be possible to measure the general relativistic gravitational redshift, which provides additional constraints on the mass and radius of the star. Owing to the long exposure, and the high efficiency and spectral resolving power of the RGS, this EXO0748–676 dataset is by far the most sensitive to date for conducting such a search.

Data were processed with the XMM-Newton Science Analysis Software (SAS) that is currently available as version 5.3.3. The soft-X-ray light curve of EXO0748–676 shows considerable variability¹³. Searching for X-ray bursts in the RGS light curve, we considered only events which conformed to the step rise/exponential decay shape characteristic of type I X-ray bursts¹⁴. We identified 28 bursts in the RGS data. The bursts varied in peak intensity between 4 counts s^{–1} and 12 counts s^{–1} with an average of 8.8 counts s^{–1}.

This represents an increase by a factor of ~ 15 over the quiescent levels observed in the periods of low activity. We defined the onset of a burst as the time at which the count rate first rises above the quiescent level by a factor of two or more. The end, less well defined, occurs when the count rate drops back to the local average level. Most bursts ranged in duration from 48 to 128 s, with an average of 90 s. Seven of the bursts were longer, with durations between 176 and 320 s.

We then extracted the first-order RGS spectra for each burst. The spacecraft pointing was stable during observations on each of the separate revolutions, but differed between revolutions by up to 40 arcsec. To generate the average burst spectrum we therefore combined the data for all observations within a single revolution, but generated separate spectral files and response matrices for each revolution. All spectral fitting was performed simultaneously on these separate data sets. For ease of display we generated a flux spectrum of the average burst, using the effective area curves for each separate data set. This allowed us to combine the data from the two RGS instruments as well. The wavelength scale is accurate to ~ 10 mÅ. The effective area is accurate to 5% for all wavelengths longer than 8 Å (ref. 10). Background subtraction was performed using the same extraction algorithms, but over the image region not occupied by the source. The background flux is a significant fraction of the total flux for wavelengths longer than ~ 32 Å. We therefore considered only the wavelength range from 8 to 32 Å in our analysis.

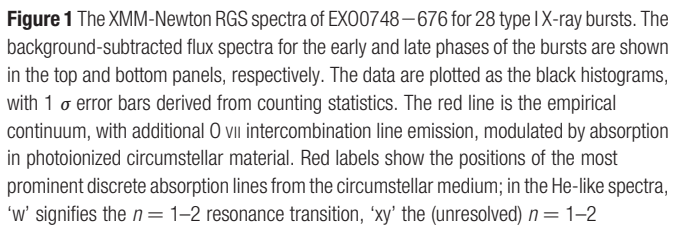
To constrain the broadband properties of the burst spectrum we examined the EPIC data. Pile-up during the bright bursts contaminated all but 250 s, or three bursts. The EPIC pn-CCD spectrum of these three bursts is well fitted by a black body, with a peak colour temperature of $kT_{\text{BB}} \approx 1.8$ keV, decaying to $kT_{\text{BB}} \leq 1.5$ keV. As the spectral properties clearly evolve during the bursts, we investigated the RGS spectrum of the early, bright phases and the decay phases separately. We explored a variety of ways to subdivide the bursts, but found that the results were not sensitive to the exact criterion used to compile the ‘early’- and ‘late’-time burst spectra. We therefore chose to divide the bursts in the simplest way, by splitting them in half by duration. The resulting flux spectra for the early and late phases of the averaged burst are shown in Fig. 1.

As in the case of the quiescent spectrum¹³, there is clear evidence for absorption and emission from highly ionized gas surrounding the neutron star during the bursts. O VII K-shell emission (consisting of $n = 1$ –2 resonance, intercombination and forbidden lines at 21.60, 21.80 and 22.10 Å) are clearly detected, as is absorption by O VII (photoelectric absorption at 16.78 Å, resonance line absorption at 21.60 Å). The fact that the O VII line emission is dominated by the intercombination transition indicates that the gas is recombining, which implies that the ionization is driven by photoionization, and that the electron density is relatively high ($n_e \geq 10^{12}$ cm^{–3}). We saw a clear change in the nature of the O VII spectrum as the bursts progressed, with the emission weakening and the absorption increasing from the early to the late phases, indicative of an overall progressive flattening of the source geometry towards the line of sight. Wavelength shifts in the O VII absorption features indicate a significant bulk outflow velocity of $v \approx 5,000$ km s^{–1} during the bursts.

In order to develop a physically consistent model for the spectral transmission of the circumstellar absorber, which we need in order to quantitatively account for its contribution to the observed absorption spectra, we fitted the O VII and O VIII spectra and measured the intensity, velocity width and Doppler shifts of the emission lines, and the ion-column density and velocity broadening of the absorption features for both the early- and late-phase spectra. The model assumes an empirical continuum spectrum during the bursts, constructed from a black body and a power law, whose parameters were optimized by eye. O VII and O VIII absorption spectra were calculated with a spectral code originally developed to interpret the X-ray absorption spectra of active galactic nuclei

We then examined the spectrum for any remaining structure that is not associated with the circumstellar absorber. In the early-time

Neither do the features match the wavelengths of absorption lines



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expected from the circumstellar absorber, with the possible exception of the 26.4 Å feature in the late-phase spectrum, which is probably partly due to C VI Lyman γ (Ly γ) at 26.6 Å. We examined absorption models appropriate to higher ionization parameters (over the range $\xi = 10$ –100) and found that the features cannot be made consistent with circumstellar absorption at any ionization parameter. This explanation can be rejected on purely spectroscopic grounds: it requires significant velocity shifts that vary randomly between ions that are present at similar ionization parameters. Furthermore, fitting individual absorption lines to the features produced serious inconsistencies in terms of the predicted overall absorption structure due to any given single ion. Finally, the features do not appear in the (accretion-dominated) quiescent spectrum of the source.

We are left with the possibility that the absorption features arise in the photosphere of the neutron star. The magnetic fields in the neutron stars in low-mass X-ray binaries are believed to be small^{19–21}, so that field effects do not affect the atomic structure, and we can use well-established atomic spectroscopy to interpret the wavelengths. In the only other example of non-trivial structure in the spectrum of a neutron star²², the correct spectroscopic identification, and hence the inferred redshift, depends critically on the unknown strength of the stellar magnetic field. We note that the previous detections of large equivalent-width features in the 4–5 keV band with proportional counters²³ have not been substantiated by subsequent observations²⁴, and that these and other similar detections^{25,26} are most probably due to instrumental effects.

We have examined all the spectra of the K-shell ions of C through to and including Si, and find no multiple coincidences between observed and predicted line positions, with identical redshifts for all ions. However, an important clue to identifying the features lies in the presence of the 13.0 Å line at early times and the presence of the 13.75 Å line at late times. A solution to the Saha ionization balance for densities $n \approx 10^{23} \text{ cm}^{-3}$ (as expected in a neutron star atmosphere at a Rosseland optical depth of unity, above which most of the absorption spectrum is formed) indicates that iron should be primarily in its H-like charge state at temperatures $kT \geq 1.2 \text{ keV}$, whereas at $kT \leq 1.2 \text{ keV}$, the He-like charge state dominates; these temperatures roughly correspond to the observed colour temperature early and late in the bursts, respectively. At these temperatures, the ionization balance of iron does not shift into the fully stripped charge state until the density drops below $\sim 10^{22} \text{ cm}^{-3}$. The density and temperature in a real atmosphere exhibit significant gradients, but the Saha balance at a representative temperature and density usually provides a good indication as to which ions of a given element are likely to be dominant in the stellar absorption spectrum. The $n = 2$ –3 transitions in the H-like ion (the analogue of the H α Balmer line) occur at 9.518, 9.533, 9.579 and 9.675, 9.690, 9.738 Å (ref. 27). Identifying these transitions with the feature at 13.0 Å in the early-phase burst spectrum implies a redshift of $z = 0.35$. The strongest $n = 2$ –3 transitions in the He-like ion occur at 10.213, 10.048 Å (E. Behar, personal communication; the 10.213 Å transition has the highest oscillator strength). Identifying these transitions with the feature at 13.75 Å in the late-phase spectrum also implies a redshift of $z = 0.35$. Members of higher-order series will all lie at wavelengths lower than 10 Å, to which our spectrum is not sensitive.

The Saha balance at temperatures $kT \geq 1 \text{ keV}$ and densities $n \approx 10^{23} \text{ cm}^{-3}$ indicates that all the lighter elements should be nearly stripped, so we would not expect to see their K-shell absorption lines, with the possible exception of oxygen, in view of its relatively high abundance. Applying the same redshift to the O VIII Ly α line (rest wavelength 18.97 Å) we would expect to see a feature at 25.6 Å. We speculate that the double (wavelengths 25.2 and 26.4 Å) structure observed at late phases, which is centred at this wavelength, is in fact a self-reversed, broad O VIII Ly α line, where the self-reversed profile is indicative of extended structure to the outer

atmosphere, and possibly a slow outflow, such as observed in the strong ultraviolet resonance lines in massive stars with extended atmospheres²⁸. The corresponding O VIII Lyman β (Ly β) line (21.60 Å at $z = 0.35$) would be hidden in the O VII spectrum from the circumstellar medium. The remaining features are all of relatively low significance, have no obvious spectroscopic interpretation, and are probably statistical fluctuations. A quantitative interpretation of the strength and shape of the features we have identified will have to await a dedicated full model-atmosphere calculation, which may have to incorporate effects induced by the X-ray bursts.

We have identified three sets of redshifted transitions in iron and oxygen in the EXO0748–676 spectrum, all with an implied redshift of $z = 0.35$. We have compared our result with the family of theoretical mass–radius relations in ref. 6. A redshift of $z = 0.35$ is consistent with most modern equations of state for neutron stars composed of normal matter in the mass range of $M \approx 1.4$ –1.8 solar masses and in the radius range of $R \approx 9$ –12 km, depending on the choice of M – R relation. This is compatible with a neutron star with a birth mass near the average for non-accreting neutron stars ($M = 1.4$ solar masses), that has been accreting mass at the observed rate for $\sim 10^9$ years, and agrees with the estimated masses of other accreting neutron stars^{2–4}. A redshift of $z = 0.35$ is inconsistent with several M – R relations based on equations of state for more exotic matter such as strange quark matter or kaon condensates^{6,7}, unless, for some of these equations of state, the mass of the neutron star in EXO0748–676 is ≤ 1.1 solar masses, which is lower than predicted by astrophysical arguments. $\square$

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Pressure-induced crystallization of a spin liquid

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Liquids are expected to crystallize at low temperature. The only exception is helium, which can remain liquid at 0 K, owing to quantum fluctuations^{1,2}. Similarly, the atomic magnetic moments (spins) in a magnet are expected to order at a temperature scale set by the Curie–Weiss temperature θ_{CW} (ref. 3). Geometrically frustrated magnets represent an exception. In these systems, the pairwise spin interactions cannot be simultaneously minimized because of the lattice symmetry⁴. This can stabilize a liquid-like state of short-range-ordered fluctuating moments well below θ_{CW} (refs 5–7). Here we use neutron scattering to observe the spin liquid state in a geometrically frustrated system, $Tb_2Ti_2O_7$, under conditions of high pressure (~ 9 GPa) and low temperature (~ 1 K). This compound is a three-dimensional magnet with $\theta_{CW} = -19$ K, where the negative value indicates antiferromagnetic interactions. At ambient pressure $Tb_2Ti_2O_7$ remains in a spin liquid state down to at least 70 mK (ref. 8). But we find that, under high pressure, the spins start to order or ‘crystallize’ below 2.1 K, with antiferromagnetic order coexisting with liquid-like fluctuations. These results indicate that a spin liquid/solid mixture can be induced by pressure in geometrically frustrated systems.

$Tb_2Ti_2O_7$ is an insulating pyrochlore oxide in which localized Tb^{3+} spins occupy a lattice of corner-linked tetrahedra. Antiferromagnetic Heisenberg interactions on this lattice are highly frustrated, giving rise to macroscopic degeneracy in the ground state⁴. The pyrochlore lattice can also be frustrated for ferromagnetic interactions with a strong local Ising anisotropy, leading to ‘spin ice’ behaviour⁹. In general, the degeneracy of the magnetic ground states may be lifted by perturbations arising from chemical disorder

or additional magnetic interactions. These include further-neighbour exchange⁴, anisotropy¹⁰ and dipolar interactions¹¹. Consequently, most antiferromagnetic pyrochlores undergo either spin-glass-like or long-range-ordering transitions^{10,12–14}. $Tb_2Ti_2O_7$ is the only one that remains in a fluctuating paramagnetic state down to 70 mK (ref. 8), although spin glass behaviour below 70 mK has been claimed¹⁵. Muon spin relaxation⁸ and neutron scattering^{8,16,17} show that the Tb spins begin to develop short-range antiferromagnetic correlations below 100 K.

The single-ion ground state in $Tb_2Ti_2O_7$ is a crystal-field doublet, which interacts with its neighbours via superexchange and dipolar coupling. Given these simple interactions, the absence of magnetic order is surprising^{8,16,18}. Further interactions, such as mixing with higher crystal-field states, may therefore play a role in stabilizing the spin liquid state¹⁹. Applied pressure offers an opportunity to study this stability, by perturbing the balance of the various interactions, which have different dependences on interatomic distance.

Neutron diffraction is the only way to obtain full information about the microscopic spin arrangement. We used Kurchatov-LLB (Laboratoire Léon Brillouin) pressure cells with sapphire anvils, the only apparatus that allows neutron scattering at both very low temperatures (1.4 K) and very high pressures, $P < 10$ GPa (ref. 20). NaCl as the pressure-transmitting medium provides a quasi-hydrostatic pressure, with a non-uniform component estimated to be less than 5%. The pressure was measured by the ruby fluorescence technique with a precision of ± 0.1 GPa. The neutron diffraction spectra were recorded on the specialized high-pressure powder

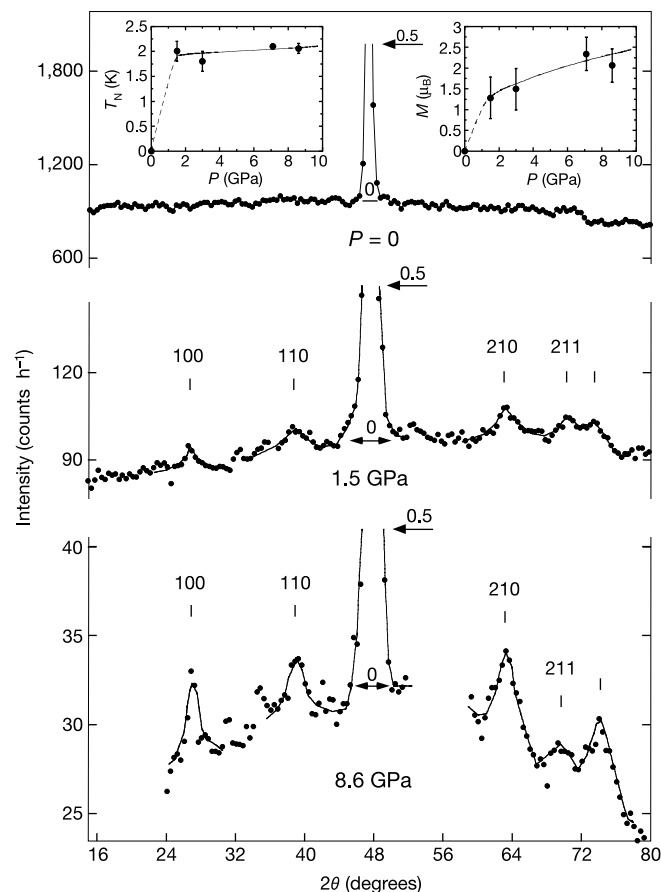


Figure 1 $Tb_2Ti_2O_7$: raw neutron diffraction spectra (neutron counts per hour) for three pressures P at 1.4 K. The incident neutron wavelength is 4.741 Å. Intensity scales are chosen to show the magnetic peaks as compared with the 111 structural peak. Half intensity of the 111 peak is shown at the centre of the spectra. Insets: the Néel temperature, T_N (left), and ordered magnetic moment at 1.4 K, M (right), versus pressure.

diffractometer G6-1 of the Laboratoire Léon Brillouin²¹, allowing detection of a neutron signal from a sample volume of less than 0.2 mm^3 . The $\text{Tb}_2\text{Ti}_2\text{O}_7$ polycrystalline sample was prepared and characterized as in ref. 8. At 8.6 GPa, the lattice constant, $a = 10.149 \text{ \AA}$ at ambient pressure, is decreased by $\Delta a/a = 1\%$. The crystal structure of $\text{Fd}\bar{3}\text{m}$ symmetry remains the same in the whole pressure–temperature range.

Figure 1 shows neutron diffraction spectra at 1.4 K for three pressures, focusing on the diffuse intensity in the region of the 111 nuclear peak. At $P = 0$, the diffuse intensity arising from liquid-like magnetic correlations shows no indication of magnetic long-range order. At 1.5 GPa, small magnetic Bragg peaks start to emerge from the diffuse background. At 8.6 GPa, the average intensity becomes much lower, but the magnetic peaks are now very clearly seen, their intensity reaching more than 30% of the 111 peak intensity. Concomitantly, the diffuse intensity shows a stronger modulation. The onset of narrow Bragg peaks shows the development of magnetic long-range order, or crystallization of the spin liquid state, induced by pressure. Applying very high pressures allows us to show this effect unambiguously, and to study it in detail versus temperature with good precision.

Figure 2a shows raw neutron diffraction spectra at 7.1 GPa for three temperatures. The magnetic Bragg peaks start to develop

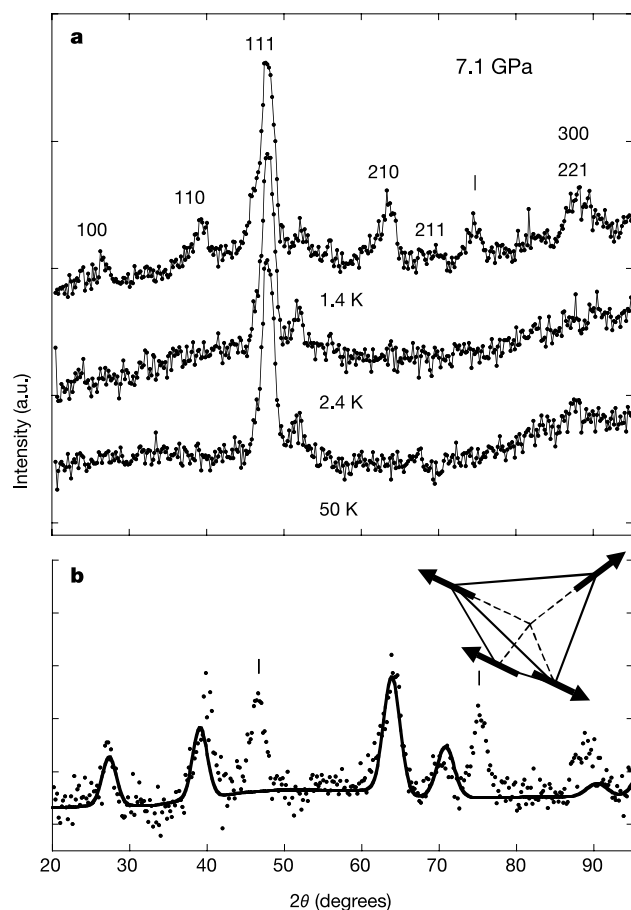


Figure 2 $\text{Tb}_2\text{Ti}_2\text{O}_7$: neutron diffraction spectra at $P = 7.1 \text{ GPa}$. **a**, Raw data at $T = 1.4$, 2.4 and 50 K . **b**, Magnetic neutron diffraction spectrum at 1.4 K . A spectrum averaged over all temperatures in the paramagnetic regime ($T > 2.1 \text{ K}$) has been subtracted. The solid line is the best refinement, corresponding to the local spin arrangement drawn in the inset. Here, the four spins are directed along $\langle 111 \rangle$ axes of the tetrahedron. One spin is along a local anisotropy axis which connects the summit to the centre, while three spins are collinear and along a different $\langle 111 \rangle$ axis. Within the unit cell, two tetrahedra have the same spin orientations, and the other two have spins reversed. The peaks at 47° and 75° correspond to a magnetic order involving a larger magnetic cell.

below the Néel temperature $T_N = 2.1 \text{ K}$ (Fig. 3 inset). The value of T_N is almost pressure independent in the investigated pressure range (Fig. 1 inset). The width of the magnetic peaks is limited by the experimental resolution ($\Delta d/d = 1.1\%$), yielding a minimum correlation length of $450 \text{ \AA}$. The liquid-like correlations start to develop below about 50 K . They persist below T_N , showing that the liquid and ordered states coexist.

The ordered magnetic structure has a simple cubic unit cell, derived from the chemical one of $\text{Fd}\bar{3}\text{m}$ symmetry by a propagation vector $\mathbf{k} = 100$ or 110 . Two extra magnetic peaks can only be indexed on a much larger unit cell, suggesting a long-wavelength modulation of the main structure. There is no magnetic contribution to the structural peaks. The magnetic structure is not predicted by theoretical models^{4,11,13} and differs from the few ordered structures reported in pyrochlore compounds, which have propagation vectors $\mathbf{k} = 0$ or $1/2 \ 1/2 \ 1/2$ (refs 10, 12, 13).

To study precisely the spin arrangement in the unit cell, the peak intensities of the main structure were refined using the Rietveld method with the FullProf program²². A relatively good agreement was found with the spin arrangement described in Fig. 2b. We tried systematically all spin arrangements along principal directions allowed by a symmetry analysis (to be described elsewhere), without finding a better solution. The ordered moment of the Tb^{3+} ion increases with increasing pressure (Fig. 1 inset), reaching a value of $2.4 \mu_B$ at 7.1 GPa and 1.4 K , where μ_B is the Bohr magneton. It seems to saturate in the high pressure range. Note that this moment remains well below the local free ion value of $9 \mu_B$, and below the $5 \mu_B$ value calculated for $\text{Tb}_2\text{Ti}_2\text{O}_7$ (ref. 16). This is expected, owing to the coexistence of liquid and ordered states.

Plotting the modulation amplitude of the diffuse scattering $A(P, T)$, where P and T are respectively pressure and temperature, shows clear evidence of this coexistence. $A(P, T)$ is defined experimentally (Fig. 3) as $I_{\text{max}} - I_{\text{min}}$, where I_{max} and I_{min} are the extrema of the diffuse intensity for a given scattering pattern. It is expected to be proportional to the thermal average of the first-neighbour spin correlations^{8,23}. A is found to increase with decreasing temperature, and this effect becomes more pronounced as pressure increases. The onset of long-range order at T_N coincides with a sharp kink of A . Below T_N , the decrease of A mirrors the increase of the Bragg intensity, showing that spin liquid and ordered

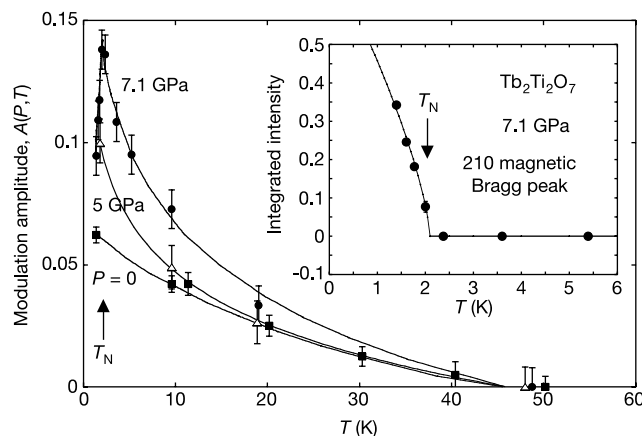


Figure 3 Temperature dependence of the modulation amplitude $A(P, T)$ for the pressures $P = 0$, 5 and 7.1 GPa , where $A(P, T) = I_{\text{max}} - I_{\text{min}}$, the difference in extrema of the diffuse intensity in our experimental range. To compare data at different pressures, $A(P, T)$ is scaled to the integrated intensity of the 111 Bragg peak. At 5 and 7.1 GPa , solid lines above T_N are fits with the law $-\ln(T/T_{\text{up}})$, $T_{\text{up}} = 46 \text{ K}$ (see text). At 5 GPa , only the paramagnetic regime was investigated. Inset, integrated intensity of the 210 magnetic peak (scaled to the 111 peak intensity) versus temperature at 7.1 GPa . The solid line is a guide to the eye.

phases coexist down to at least 1.4 K ($0.7T_N$). Therefore the magnetic state below T_N is a mixed spin liquid / spin solid phase, with both static and dynamical character, and a gradual transfer of intensity from the liquid state to the ordered state as temperature decreases. The question of whether the two states still coexist at $T = 0$ remains open, as we observe no saturation of the Bragg intensity at 1.4 K.

The coexistence of spin liquid and ordered states and the fact that T_N is pressure independent suggest that the pressure-induced transition may be of first order. It is reminiscent of other geometrically frustrated magnets at ambient pressure. $\text{Gd}_3\text{Ga}_5\text{O}_{12}$ exhibits a transition from spin liquid to antiferromagnetic order in a finite window of applied field, which has been compared to the phase diagram of liquid helium^{6,24}. In $\text{Gd}_3\text{Ga}_5\text{O}_{12}$ the spin liquid regions were found to coexist with either spin glass or medium ordered regions^{6,7}, possibly induced by Gd:Ga site mixing. In $\text{SrCr}_9\text{pGa}_{12-9\text{p}}\text{O}_{19}$, well-defined quantum states of isolated Cr^{3+} spin pairs were identified, the sample again displaying both spin liquid and spin glass features. The present data, where magnetic order is induced by pressure in a fully chemically ordered sample, confirm that this behaviour may be typical of geometrically frustrated magnets.

The spin liquid state under pressure shows other important peculiarities. Using the procedure described in Fig. 4 legend, we show that data at different pressures and temperatures all superimpose, but differ from the data at ambient pressure. In particular, the diffuse peak centred at $P = 0$ at the 111 peak position of the crystal structure (wavevector transfer in reduced units $qa = 10.9 \pm 0.1$) shifts under pressure to a higher qa value of 12.6 ± 0.3 (here, q is the wavevector transfer and a is the lattice constant). To investigate this effect, the magnetic diffuse scattering was fitted by a liquid-like function $I(q) = -B(T)/f^2(q) \sin(qR_1)/(qR_1) + c(T)$, where B and c are parameters^{8,23}. B is proportional to the modulation amplitude A , whereas c is a small background intensity involving a phonon contribution. $f(q)$ is the magnetic form factor of Tb^{3+} . The position of the maximum of the diffuse intensity is fixed by the distance between nearest-neighbour Tb^{3+} ions, $R_1 = a/\sqrt{8}$. This law describes well the diffuse scattering under pressure, showing that the correlation length remains constrained to first-neighbour pairs, even very close to T_N . The variation of $A(T)$ (Fig. 3) is described well by the law $-\ln(T/T_{\text{up}})$ down to T_N , where T_{up} locates the onset of the short-range spin correlations¹⁴. In contrast with the pressure data, we

cannot fit the data at $P = 0$ with the above laws, neither for the q nor for the T dependence. Finally, it appears that under pressure the magnetic fluctuations are enhanced and their q dependence yields better agreement with a predicted liquid-like scattering function^{25,26}.

To summarize, the application of pressure in $\text{Tb}_2\text{Ti}_2\text{O}_7$ results in magnetic long-range order that coexists with spin liquid. The pressure-induced order provides further compelling evidence that the spin liquid state in $\text{Tb}_2\text{Ti}_2\text{O}_7$ is most unusual. In liquid helium, pressure induces crystallization by simply strengthening the interatomic interactions, which reduces the quantum fluctuations. Here the role of pressure is more intricate. One possibility is that the frustration is relieved by a pressure-induced structural distortion, but this has not been observed to within a precision of $\Delta a/a \approx 10^{-3}$. A more intriguing possibility is that in the spin liquid state, exchange, dipolar and crystal-field interactions are naturally balanced in such a way as to make quantum fluctuations significant. This delicate balance is then destroyed by pressure, resulting in magnetic order. Whatever the exact mechanism, by successfully measuring neutron scattering under conditions of high pressure and low temperature, we have revealed an unusual property of a spin liquid system. This result might well hold the key to understanding this unusual magnetic state. □

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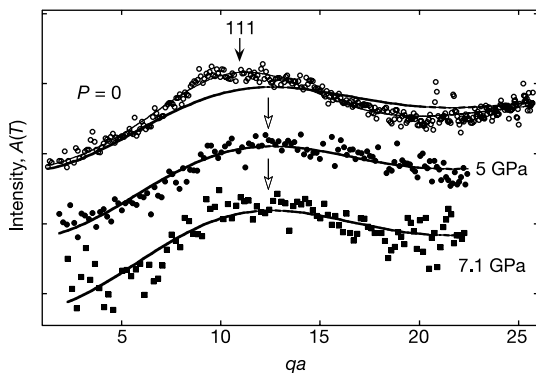


Figure 4 Magnetic diffuse intensity in the spin liquid state at $P = 0$, 5 and 7.1 GPa. The neutron spectra are corrected from a spectrum measured at 50 K. The intensities, scaled to the modulation amplitude, are plotted versus the wavevector transfer qa in reduced units. This procedure allows all data to be compared quantitatively, and to distinguish the effect of pressure from casual lattice contraction. Solid lines are fits with the function described in the text. The thin line for $P = 0$ is a guide to the eye. Filled and open arrows show the positions of the 111 peak and the maximum of the fitted law, respectively.

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Epitaxial core-shell and core-multishell nanowire heterostructures

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Semiconductor heterostructures with modulated composition and/or doping enable passivation of interfaces and the generation of devices with diverse functions¹. In this regard, the control of interfaces in nanoscale building blocks with high surface area will be increasingly important in the assembly of electronic and photonic devices^{2–10}. Core-shell heterostructures formed by the growth of crystalline overlayers on nanocrystals offer enhanced emission efficiency⁷, important for various applications^{8–10}. Axial heterostructures have also been formed by a one-dimensional modulation of nanowire composition^{11–13} and doping¹¹. However, modulation of the radial composition and doping in nanowire structures has received much less attention than planar¹ and nanocrystal⁷ systems. Here we synthesize silicon and germanium core-shell and multishell nanowire heterostructures using a chemical vapour deposition method applicable to a variety of nanoscale materials¹⁴. Our investigations of the growth of boron-doped silicon shells on intrinsic silicon and silicon-silicon oxide core-shell nanowires indicate that homoepitaxy can be achieved at relatively low temperatures on clean silicon. We also demonstrate the possibility of heteroepitaxial growth of crystalline germanium-silicon and silicon-germanium core-shell structures, in which band-offsets drive hole injection into either germanium core or shell regions. Our synthesis of core-multishell structures, including a high-performance coaxially gated field-effect transistor, indicates the general potential of radial heterostructure growth for the development of nanowire-based devices.

Our approach to the synthesis of core-shell nanowire structures is based upon control of radial versus axial growth (Fig. 1). We have previously developed a general method for synthesis of semiconductor nanowires using nanocluster catalysts to direct axial growth by a vapour-liquid-solid growth process (Fig. 1a)^{15,16}. Axial growth is achieved when reactant activation and addition occurs at the catalyst site and not on the nanowire surface (Fig. 1b). Correspondingly, it is possible to drive conformal shell growth by altering conditions to favour homogeneous vapour-phase deposition on the nanowire surface (Fig. 1c). Subsequent introduction of different reactants and/or dopants produces multiple shell structures of nearly arbitrary composition, although epitaxial growth of these shells requires consideration of lattice structures. This approach to

core-shell nanowire heterostructures is elaborated below for the technologically important silicon (Si) and germanium (Ge) systems¹⁷.

Homoepitaxial Si-Si core-shell nanowires were grown by chemical vapour deposition (CVD) using silane as the silicon reactant (Fig. 2)¹⁸. Intrinsic silicon (i-Si) nanowire cores were prepared by gold-nanocluster directed axial growth, which yields single-crystal structures with diameters controlled by the nanocluster diameter, and then boron-doped (p-type) silicon (p-Si) shells were grown by homogeneous CVD, where the shell thickness was directly proportional to the growth time. Radial shell growth can be 'turned-on' by the addition of diborane, which serves both to lower the decomposition temperature of silane¹⁹ and acts as a p-type dopant. Transmission electron microscopy (TEM) images of the i-Si/p-Si product obtained from constant temperature growth shows a uniform core-shell structure consisting of a crystalline Si core and amorphous Si shell (Fig. 2a), where the core diameter, 19 nm, is consistent with the 20-nm nanocluster used in the initial axial growth step. TEM images show reproducible crystalline faceting at the core-shell interface (Fig. 2b). This faceting suggests that the nanowire surfaces are sufficiently clean following axial growth to nucleate epitaxial shell growth.

To understand and control Si on Si homoepitaxy in core-shell nanowire structures we carried out several distinct experiments. First, i-Si/p-Si core-shell nanowires prepared as above were annealed *in situ* at 600 °C. TEM images recorded on the annealed samples exhibited no diffraction contrast between the core and shell (Fig. 2c), and lattice-resolved images and electron diffraction data further show that the shell crystallizes to yield a single-crystal structure (Fig. 2d). Second, the importance of the initial nucleation for achieving epitaxy in the shell was probed using *in situ* oxidation of the silicon core (see Methods), which produces a thin amorphous silicon oxide layer at the surface of the core, before silicon-shell growth. Significantly, TEM images of i-Si/SiO_x/p-Si core-shell-shell structures show a smooth and abrupt interface between the crystalline core and amorphous shell (Fig. 2e, f). The low roughness of the interface is comparable to that observed in nanowires after only axial growth, and contrasts sharply with the faceted interface of the low-temperature homoepitaxy (Fig. 2b). These results show that the thin oxide layer completely disrupts homoepitaxy; further annealing and TEM studies show that oxidation inhibits crystallization of

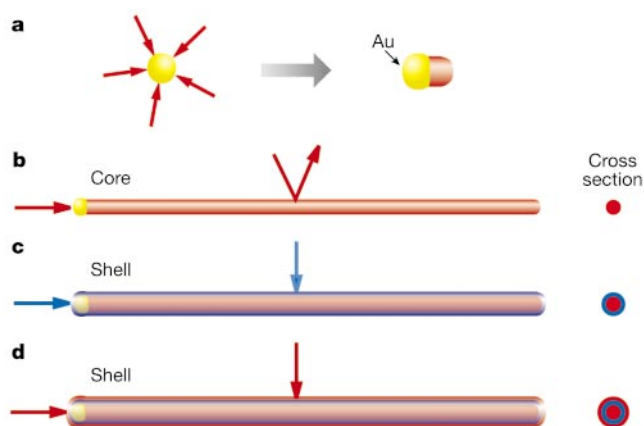


Figure 1 Synthesis of core-shell nanowires by chemical vapour deposition. **a**, Gaseous reactants (red) catalytically decompose on the surface of a gold nanocluster leading to nucleation and directed nanowire growth. **b**, One-dimensional growth is maintained as reactant decomposition on the gold catalyst is strongly preferred. **c**, Synthetic conditions are altered to induce homogeneous reactant decomposition on the nanowire surface, leading to a thin, uniform shell (blue). **d**, Multiple shells are grown by repeated modulation of reactants.

the shell under annealing conditions that lead to complete crystallization in samples without the oxide layer.

We have used three-terminal measurements to characterize the electrical transport properties of the three distinct types of i-Si/p-Si core-shell structures, thereby defining the impact of the observed structural differences (Fig. 2g). First, the i-Si/SiO_x/p-Si nanowires (Fig. 2f), which have an amorphous p-Si shell, show relatively high resistivities, about $10^3 \Omega \text{ cm}$, and low hole mobilities, about $0.001 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. These resistivity and mobility values are comparable to those of heavily doped amorphous silicon deposited by plasma-enhanced CVD²⁰, and suggest that conduction is dominated by the amorphous p-Si shell. In contrast, the i-Si/p-Si structures, which have partly or fully crystalline p-Si shells, exhibit much lower resistivities, about $(0.5\text{--}5) \times 10^{-3} \Omega \text{ cm}$. The similarity in these values is consistent with our observation of crystalline faceting (Fig. 2b), and suggests that a continuous epitaxial layer of p-Si, which dominates transport, exists prior to complete crystallization by annealing. In addition, the hole mobility of the crystalline p-Si shell nanowires, $25 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$, is comparable to that of single-crystal silicon at similar high doping levels (see Supplementary Materials)²¹.

Radial heteroepitaxy of Si on Ge was pursued to explore the potential of our approach to core-shell structures in materials of rapidly increasing scientific and technological importance¹⁷. For example, the energy band offsets in Si-Ge heterostructures produce internal fields that drive charge-carrier redistribution²², which can enable high-mobility devices. Single-crystal Ge nanowires were

defined through gold nanocluster directed axial growth, and then boron-doped p-Si shells were grown by homogeneous CVD (see Methods). Bright-field TEM images (Fig. 3a) reveal a core-shell structure that is consistent with Ge-core (dark) and Si-shell (light) structure, and we confirmed this assignment by elemental mapping (Fig. 3b, c), which shows a localized Ge core and Si shell. High-resolution TEM images of i-Ge/p-Si core-shell nanowires in which the p-Si shell was deposited at low temperature without annealing show a crystalline Ge core and predominantly amorphous Si shell (Fig. 3d). Analysis of cross-sectional elemental mapping data (Fig. 3e) shows that the Ge core is about 26 nm, the Si shell is about 15 nm, and the Ge-Si interface width is believed to be $<1 \text{ nm}$ on the basis of the electron beam width and modelling (see Methods).

Significantly, we find that the amorphous Si shell can be completely crystallized following *in situ* thermal annealing at 600°C . Lattice-resolved TEM images of Ge-Si core-shell structures following this thermal treatment exhibit a uniform crystalline Si shell (Fig. 3f), and suggest that thin regions of epitaxially grown Si are present in the unannealed wires. Higher silicon-deposition temperatures might make the annealing step unnecessary by improving the surface mobility of adsorbed silicon. Elemental mapping (Fig. 3g) confirms that the contrast in high-resolution TEM images is consistent with an abrupt ($<1 \text{ nm}$) Si-Ge interface. Although the present measurements cannot rule out some interface mixing, previous studies of planar growth under similar conditions found no evidence for Si-Ge interdiffusion²³. Notably, preliminary elec-

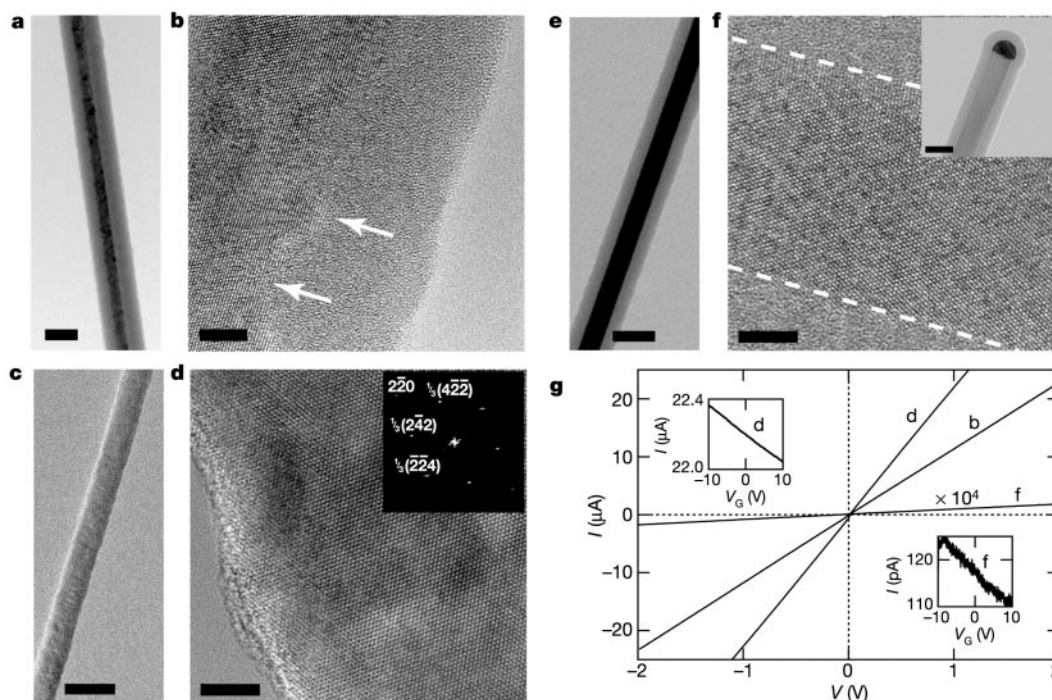


Figure 2 Si-Si homoepitaxial core-shell nanowires. **a, b**, Diffraction contrast and high-resolution TEM images, respectively, of an unannealed intrinsic silicon core and p-type silicon shell nanowire grown at 450°C . Crystal facets in the high-resolution TEM image designated by arrows indicate initially epitaxial shell growth at low temperature. Scale bars are 50 nm and 5 nm, respectively. **c, d**, TEM images (analogous to **a** and **b**) of an i-Si/p-Si core-shell nanowire annealed at 600°C for 30 min after core-shell growth at 450°C . Inset, two-dimensional Fourier transforms of the image depicting the $[111]$ zone axis of the single crystal nanowire. The $\frac{1}{3}\{422\}$ reflections, although forbidden in bulk silicon, arise as a result of the finite thickness of the nanowire²⁷. **e, f**, TEM images of an i-Si/SiO_x/p-Si nanowire. The oxide layer is too thin ($<1 \text{ nm}$) to discern in the high-

resolution image, but the sharp interface (dashed line) between the crystalline core and amorphous overcoat clearly differs from the faceting seen in **b** and illustrates the disruption of epitaxy. Inset, TEM image of p-Si coating the nanowire and the Au nanocluster tip. Scale bar is 50 nm. **g**, Three-terminal current (I) versus voltage (V) measurements of the nanowires described in the preceding panels. A schematic of the measurement set-up is provided in the Supplementary Information. Curves b, d and f are labelled according to the representative TEM image (**b**, **d** and **f**) from the same sample of wires. Curve f has been multiplied by a factor of 10^4 . Insets, current versus backgate voltage to determine field-effect mobilities (see Supplementary Information).

trical transport studies of the i-Ge/p-Si core-shell nanowires provide good evidence for internal field-driven carrier redistribution (see Supplementary Information), and we believe these systems are worth investigating further.

Synthetic control of Si-Ge core-multishell nanowire structures could be used to explore a variety of fundamental phenomena and new device concepts, although achieving this goal will require the ability to prepare an essentially arbitrary sequence of Si, Ge and alloy overlayers on both Si and Ge nanowire cores. To this end, we studied Ge deposition on Si nanowire cores. TEM images and composition mapping (Fig. 4a) show the Si-Ge core-shell structure with a sharp (<1 nm) interface, and demonstrate that the Ge shell is fully crystallized for low-temperature growth (Fig. 4b), presumably owing to the higher surface mobility of Ge adatoms. In addition, diffraction data are consistent with coherently strained epitaxial overgrowth (inset Fig. 4b); that is, a single diffraction peak is observed along the axial direction, which indicates compressively strained Ge and tensile-strained Si. Two peaks, which can be indexed to the Ge (5.657 Å) and Si (5.431 Å) lattice constants, are also observed in the radial direction and indicate relaxation normal to the interface. Preliminary transport studies of these structures (Supplementary Information) provide evidence for hole injection from the p-Si core to i-Ge shell as expected from valence band offsets²². Finally, we have also explored the growth of more complex multishell structures; composition mapping of a Si-Ge-Si core-double-shell structure (Fig. 4c) demonstrates the potential of extending our approach in this direction.

Lastly, we have used our approach to prepare new device structures, such as a coaxially gated nanowire field-effect transistor (FET) (Fig. 5). The coaxial geometry offers advantages for nanofabrication, such as a capacitance enhancement compared to standard planar gates used in nanowire³ and nanotube²⁴ FETs, and might be

best compared to double-gated structures being investigated for advanced planar devices²⁵. The nanowire building blocks used to fabricate coaxial FETs consisted of a core-multishell structure: p-Si/i-Ge/SiO_x/p-Ge, where the active channel is the i-Ge shell. The source, drain and gate contacts were made by selective etching and metal deposition onto the inner i-Ge shell and outer p-Ge shell, respectively. Significantly, transport measurements made on these initial devices show very good performance characteristics (Fig. 5c) with transconductance values up to $1,500 \text{ nA V}^{-1}$ for a 1-V source-drain bias. These data for unoptimized devices are comparable to recent results reported for intensively studied carbon-nanotube FETs. We are encouraged by this comparison because the values for the coaxial nanowire FET thus probably represent a lower limit to what may be achieved. For example, minimizing SiO_x trap states, which can compensate the applied gate voltage, reducing the gate dielectric thickness, and/or substituting high-K dielectrics should lead to improvements in transistor performance. We believe it is significant that all of these changes can be implemented during the initial synthesis stage, and thus integration of this advanced device structure is essentially no more difficult than for a single-component semiconducting nanowire or nanotube.

Thus we have shown that heterostructures in which composition and/or doping are modulated at the nanometre scale can be realized in core-shell nanowires. Much of our initial efforts have focused on demonstration of structural diversity in core-shell nanomaterials, and the promise of these new structures has already been demonstrated with the realization of a coaxial FET device. Future advances in nanowire semiconductor devices could be realized by increasing carrier mobilities. We believe that substantial increases could be achieved in modulation-doped core-shell structures in which, for example, a layer of i-Si separates the i-Ge core from a high-density dopant layer. Formation of a radial two-dimensional electron or

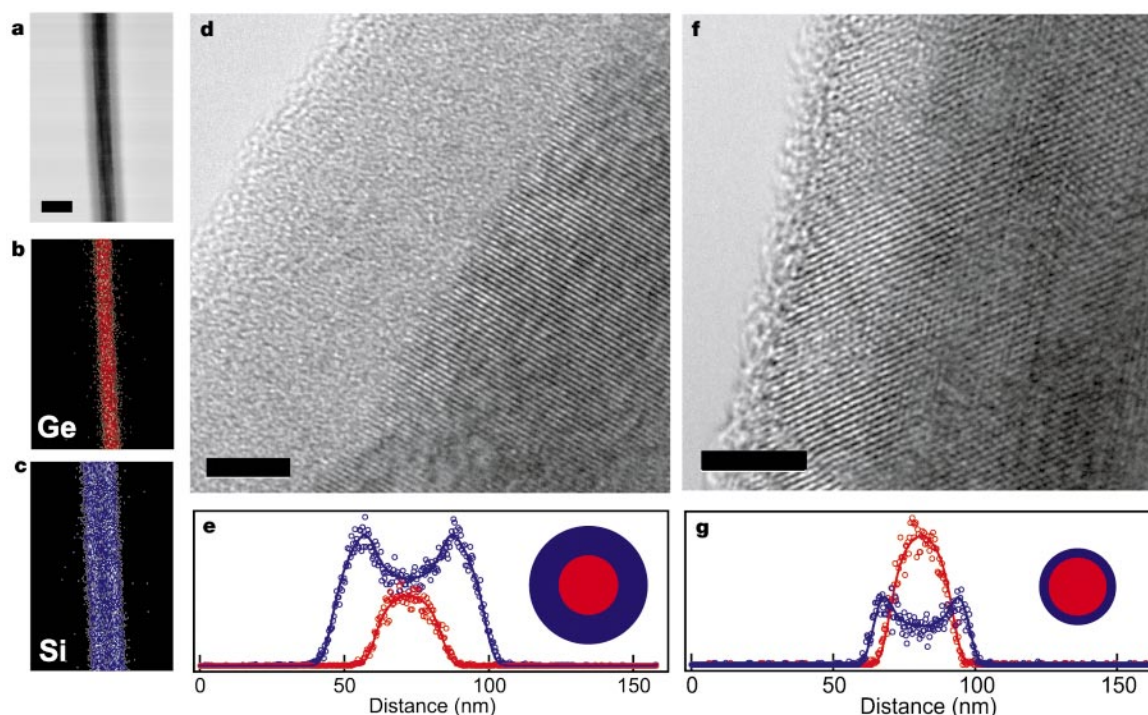


Figure 3 Ge-Si core-shell nanowires **a**, Bright-field image of an unannealed Ge-Si core-shell nanowire with an amorphous p-Si shell. Scale bar is 50 nm. **b, c**, Scanning TEM elemental maps of Ge (red) and Si (blue) concentrations, respectively, in the nanowire of **a**. **d**, High-resolution TEM image of a representative nanowire from the same synthesis as the wire in **a-c**. Scale bar is 5 nm. **e**, Elemental mapping cross-section showing the Ge (red circles) and Si (blue circles) concentrations. The solid lines show the theoretical

cross-section for a 26-nm-diameter core, 15-nm-thick shell and <1 nm interface according to the model described in the Methods section. **f**, High-resolution TEM image of an annealed Ge-Si core-shell nanowire exhibiting a crystalline p-Si shell. Scale bar is 5 nm. **g**, Elemental mapping cross-section gives a 5-nm shell thickness with a sharp interface consistent with the TEM image, suggesting that the Ge and Si do not interdiffuse substantially during the annealing process.

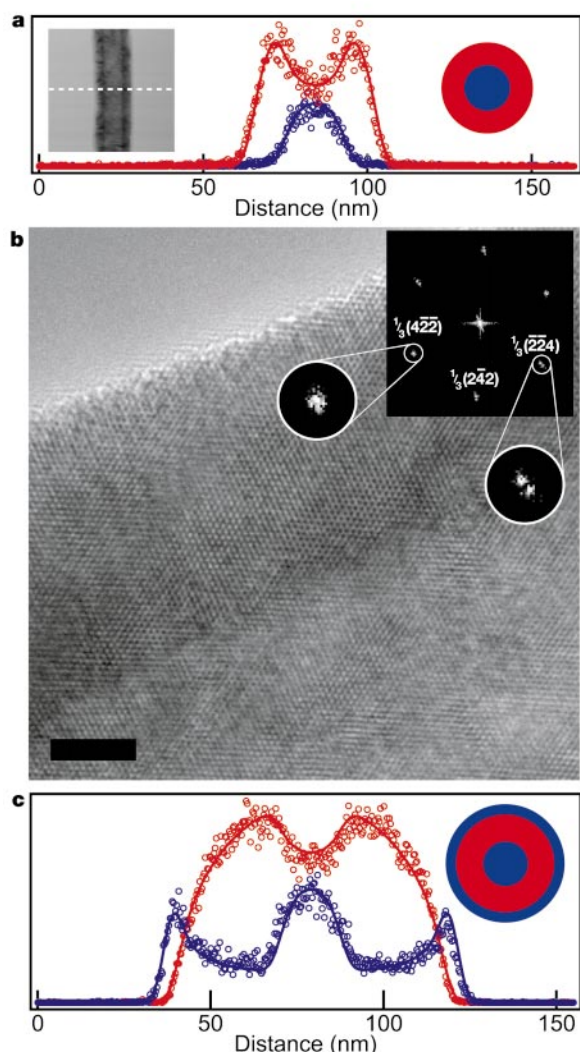


Figure 4 Si-Ge and Si-Ge-Si core-shell nanowires. **a**, Elemental mapping cross-section indicating a 21-nm-diameter Si core (blue circles), 10-nm Ge shell (red circles) and <1 nm interface. Inset, TEM image of the corresponding Si-Ge core-shell nanowire. The white dashed line indicates mapping cross-section. **b**, High-resolution TEM image of a representative crystalline nanowire core and shell from the same synthesis as the wire in **a**. Scale bar is 5 nm. Inset, two-dimensional Fourier transform of the real-space image showing the [111] zone axis. The split lattice reflections perpendicular to the interface can be indexed to the Ge and Si lattice constants (5.657 Å and 5.431 Å, respectively). **c**, Cross-sectional elemental mapping of a double-shell structure with an intrinsic silicon core (diameter, 20 nm), intrinsic germanium inner shell (thickness, 30 nm), and p-type silicon outer shell (4 nm); silicon is blue circles and germanium is red circles.

hole gas of very high mobility would significantly improve the performance of existing nanowire-based devices and open up substantial new opportunities to study electron-electron interactions in this low-dimensional system. More generally, our CVD-based epitaxial shell-growth method implies that for any active core or shell region, be it metallic, semiconducting or insulating, the surrounding material can be chosen to provide chemical passivation, charge carrier confinement, or wave-guiding capability analogous to graded-index optical fibres. When combined with the ability to produce axial nanowire heterostructures¹¹, the techniques described herein provide unprecedented control of nanometre-scale composition in two independent dimensions and represent a significant advance in the complexity and functionality of building blocks for nanoscience and nanotechnology. □

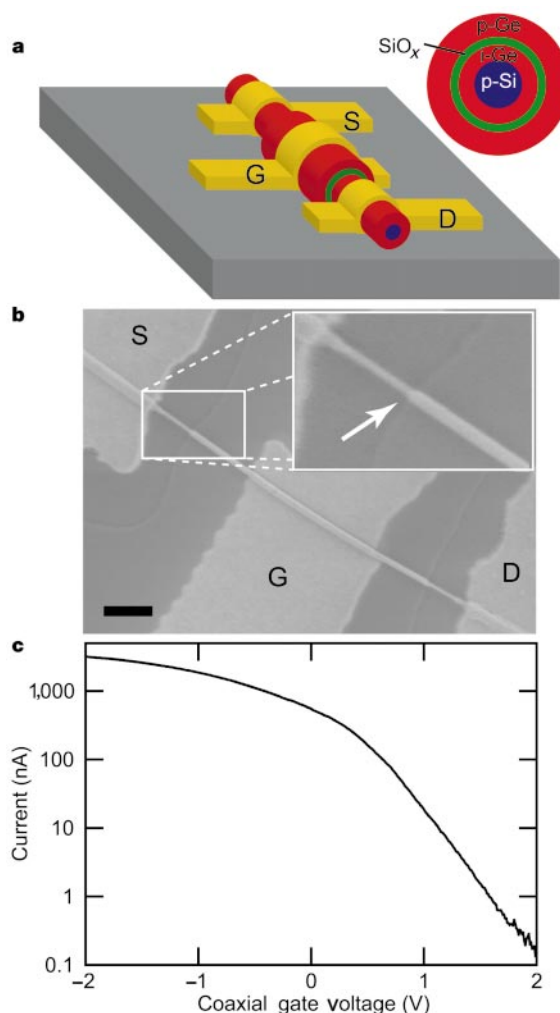


Figure 5 Coaxially-gated nanowire transistors. **a**, Device schematic showing transistor structure. The inset shows the cross-section of the as-grown nanowire, starting with a p-doped Si core (blue, 10 nm) with subsequent layers of i-Ge (red, 10 nm), SiO_x (green, 4 nm), and p-Ge (5 nm). The source (S) and drain (D) electrodes are contacted to the inner i-Ge core, while the gate electrode (G) is in contact with the outer p-Ge shell and electrically isolated from the core by the SiO_x layer. **b**, Scanning electron micrograph (SEM) of a coaxial transistor. Source and drain electrodes were deposited after etching the Ge (30% H₂O₂, 20 s) and SiO_x layers (buffered HF, 10 s) to expose the core layers. The etching of these outer layers is shown clearly in the inset and is indicated by the arrow. The gate electrodes were defined in a second step without any etching before contact deposition. Scale bar is 500 nm. **c**, Gate response of the coaxial transistor at V_{SD} = 1 V, showing a maximum transconductance of 1,500 nA V⁻¹. Charge transfer from the p-Si core to the i-Ge shell produces a highly conductive and gateable channel.

Methods

Gold nanoclusters were deposited on oxidized silicon wafers and placed in a quartz tube furnace. Silicon nanowire cores were grown at 450 °C using silane (5 cm³ at standard temperature and pressure, STP min⁻¹) at 5 torr, producing a one-dimensional (axial) growth rate of about 2 μm min⁻¹. p-type silicon shells were deposited using silane (1 cm³ STP min⁻¹) and 100 p.p.m. diborane in helium (20 cm³ STP min⁻¹) at 20 torr yielding a radial growth rate of about 10 nm min⁻¹, stoichiometric incorporation of boron would yield a bulk doping level of 2 × 10²⁰ cm⁻³, and is similar to the values obtained from transport measurements (see Supplementary Information). Ge nanowires were grown at 380 °C using 10% germane in argon (30 cm³ STP min⁻¹) at 30 torr (axial growth rate, ~0.7 μm min⁻¹), while Ge shells were deposited at 5 cm³ STP min⁻¹ and 4 torr (radial growth rate, ~10 nm min⁻¹) by changing the position of the growth substrate within the furnace. The amount of reactant that has thermally decomposed typically increases as the gas flows through the furnace, and thus radial growth may be 'turned on' by moving the growth substrate downstream to favour uncatalysed surface growth. Oxidation of i-Si cores to interrupt epitaxy was accomplished by flowing oxygen (30 cm³ STP min⁻¹) at 30 torr for 2 min. The oxide gate dielectric in the coaxially gated structures was grown at 450 °C using oxygen (2.5 cm³ STP min⁻¹) and silane (0.25 cm³ STP min⁻¹) at 1 torr for 2 min.

The substrate-bound nanowires were sonicated in ethanol and deposited on oxidized degenerately doped silicon wafers or copper grids for electrical transport and TEM measurements, respectively. Electron-beam lithography was employed to define contact regions with subsequent deposition of Ti/Au electrodes, as described previously³. Effective mobilities were calculated according to the method of ref. 26. Additional details are provided in the Supplementary Information. The integrity of the SiO₂ gate dielectric in the coaxially gated nanowire transistors was confirmed by the low, <5 pA, gate to source–drain leakage currents. The continuity of the p-Ge outer shell was verified by two-terminal resistance measurements on the shell itself.

The high-resolution TEM images were collected on a Jeol 2010F microscope, and elemental imaging and cross-sectional mapping was conducted on a VG HB603 STEM. The elemental mapping data were modelled by calculating the cross-sectional thicknesses for concentric cylinders of different composition with abrupt interfaces, taking the electron beam profile into account by convoluting with a gaussian profile of 1.6 ± 0.5 nm full-width, consistent with the known value for the instrument.

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The amount of carbon released from peat and forest fires in Indonesia during 1997

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Tropical peatlands are one of the largest near-surface reserves of terrestrial organic carbon, and hence their stability has important implications for climate change^{1–3}. In their natural state, lowland tropical peatlands support a luxuriant growth of peat swamp forest overlying peat deposits up to 20 metres thick^{4,5}. Persistent environmental change—in particular, drainage and forest clearing—threatens their stability², and makes them susceptible to fire⁶. This was demonstrated by the occurrence of widespread fires throughout the forested peatlands of Indonesia^{7–10} during the 1997 El Niño event. Here, using satellite images of a 2.5 million hectare study area in Central Kalimantan, Borneo, from before and after the 1997 fires, we calculate that 32% (0.79 Mha) of the area had burned, of which peatland accounted for 91.5% (0.73 Mha). Using ground measurements of the burn depth of peat, we estimate that 0.19–0.23 gigatonnes (Gt) of carbon were released to the atmosphere through peat combustion, with a further 0.05 Gt released from burning of the overlying vegetation. Extrapolating these estimates to Indonesia as a whole, we estimate that between 0.81 and 2.57 Gt of carbon were released to the atmosphere in 1997 as a result of burning peat and vegetation in Indonesia. This is equivalent to 13–40% of the mean annual global carbon emissions from fossil fuels, and contributed greatly to the largest annual increase in atmospheric CO₂ concentration detected since records began in 1957 (ref. 1).

In Indonesia, peatland fires are mostly anthropogenic, started by local (indigenous) and immigrant farmers as part of small-scale land clearance activities, and also, on a much larger scale, by private companies and government agencies as the principal tool for clearing forest, before establishing crops^{9,11,12}. During the abnormally long, El Niño dry season of 1997, many of these 'managed' fires spread out of control, consuming not only the surface vegetation but also the underlying peat and tree roots, contributing to the dense haze that blanketed a large part of Southeast Asia and causing both severe deterioration in air quality and health problems^{9,11}.

In order to investigate the role of peatland in the release of carbon during the 1997 fires in Indonesia, we focused on 2.5 million hectares of landscape in Central Kalimantan within a single Landsat TM (thematic mapper) image. We determined land cover types and the total area of peatland. The latter includes natural and semi-natural vegetation (peat swamp forest, PSF) and land converted to various other types of use (such as the ill-fated Mega Rice Project (MRP) that was promoted by the Indonesian government from 1995 to 1999 (Fig. 1a)). By combining peat thickness, pre-fire land cover and burnt area data we were able to estimate the amount of carbon released from peatland during the fires. Our objectives were to: (1) provide accurate information on the location and extent of

fires, (2) compare the extent of fire damage between pristine and degraded peatland, and (3) estimate the scale of carbon emissions arising from the fires. We used this information to extrapolate the probable magnitude of carbon emissions from the total peatland area in Indonesia affected by the 1997 fires.

Using Landsat TM/ETM (enhanced thematic mapper) imagery (Fig. 1b) and field checking (ground-truthing), we determined that in the 2.5-Mha study area, peatland covers 86.4% of the landscape and, before the fires (Fig. 1d, Table 1), 64.3% (1,602,413 ha) of the total area was covered by dense forest (classes 1–6) with an

additional 10.3% (257,713 ha) of forest mosaics (10–40% forest cover and >10% canopy cover¹³). Land used for agriculture and plantations occupied 14.0%, while 8.3% was bushland (fallow land with some vegetation regrowth after land clearance or fire damage) and 3.0% was swampy grassland. The land cover types on peatland were PSF (pristine, logged over and fragmented), forest mosaics, agricultural land and bushland. PSF occupied 85.3% (1,366,754 ha) of the forested area, of which 15.9% was pristine, 78.3% logged over and 5.8% fragmented (40–70% forest cover and >10% canopy¹³).

Optical satellite systems were severely hampered owing to

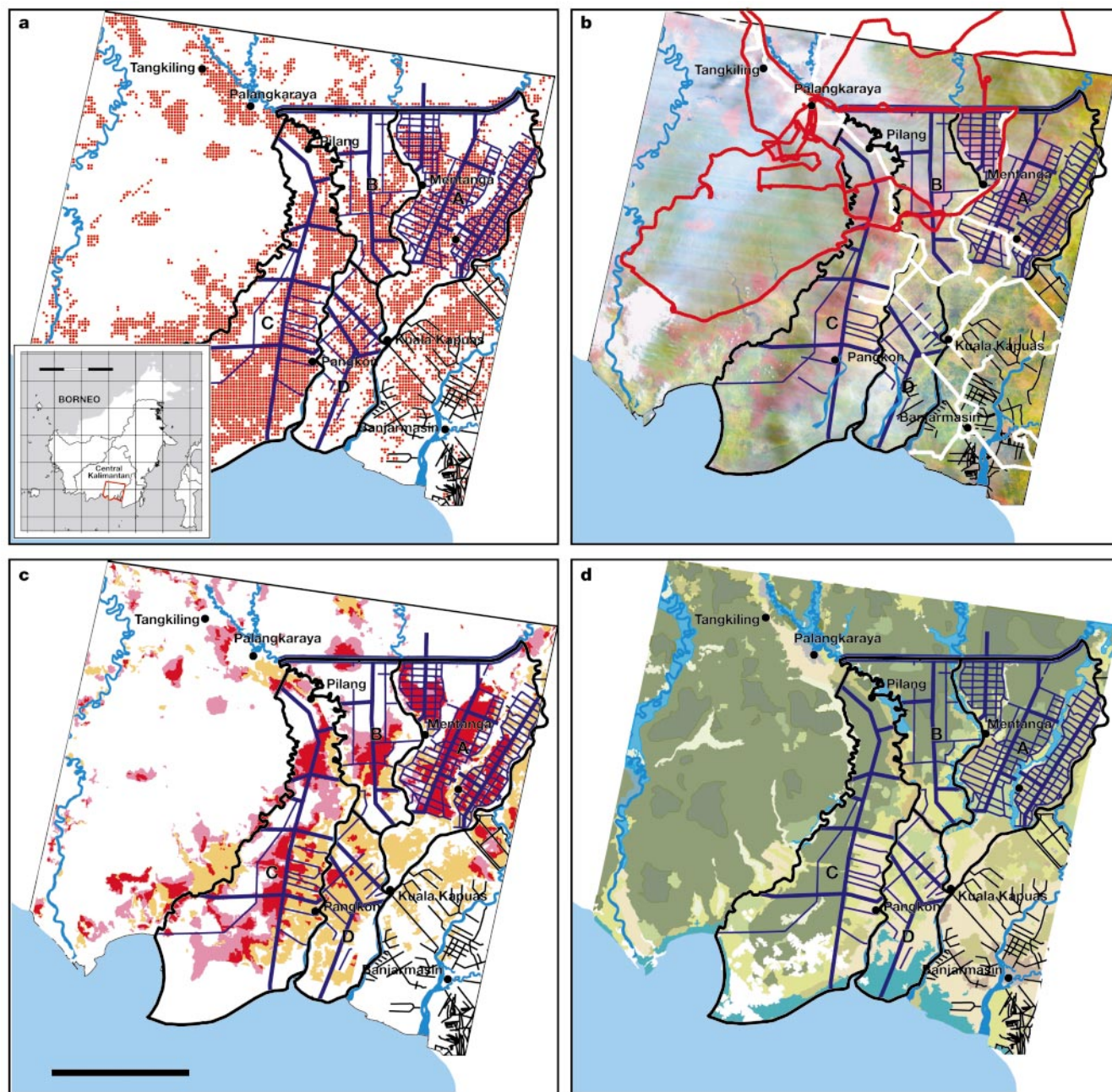


Figure 1 Study site in Central Kalimantan, Borneo, Indonesia (location see inset; bar 600 km). **a**, The four blocks of the 1-Mha MRP area (A–D) are indicated by black outlines; irrigation canals constructed between 1996 and 1997 are indicated in blue; superimposed (red dots) are NOAA AVHRR hotspots detected between June and December 1997. **b**, Post-fire Landsat TM 5 image 118-62, 29 March 1998; burn scars are visible in shades of red (RGB: TM 5 bands 5, 4, 3); superimposed are GPS-recorded ground (white) and aerial (red) surveys. **c**, Burned area derived from analysis of post-fire Landsat TM 5 and multitemporal ERS SAR images (bar 50 km); burnt areas detected by

both Landsat and ERS are indicated in red; burnt areas detected only by Landsat are shown in purple; burnt areas detected only by ERS SAR and undetectable by Landsat TM 5, owing to plant regrowth are shown in orange. **d**, Land cover map derived from pre-fire Landsat TM 5 image 118-62, 29 May 1997 (dark green, low pole peat swamp forest (PSF); green, tall PSF; beige, agriculture and fallow land; bright green, fragmented PSF and PSF mosaics; brown green, grass and bushland; blue green, mangrove forests; light blue green, pristine swamp forest (periodically inundated); pale green, dry and swampy grasslands; white, clouds; blue, rivers).

Table 1 Land cover in Central Kalimantan and the effects of the 1997 fires

Land cover types*	Total study area		Former MRP		Block C of MRP	
	Total area (10 ³ ha)	Damage in class (%)	Total area (10 ³ ha)	Damage in class (%)	Total area (10 ³ ha)	Damage in class (%)
1, Pristine PSF	217.1	4.5	19.4	14.1	1.9	99.3
2, Logged over PSF	1,070.7	29.2	373.6	56.8	179.7	56.1
3, Fragmented PSF	79.0	70.0	59.3	73.9	18.5	45.0
4, Riverine swamp forest	16.8	7.1	5.2	13.2	2.2	9.9
5, Fragmented riverine swamp forest	130.5	15.7	48.6	17.2	4.9	4.0
6, Mangrove forest	88.4	14.6	56.0	22.0	23.4	0
7, Forest mosaics	257.7	54.1	158.4	54.1	73.8	45.6
8, Bushland	207.9	45.2	111.3	55.3	17.4	46.4
9, Swampy grasslands	75.8	41.0	19.7	57.3	14.4	68.2
10, Agricultural land	321.0	36.9	132.9	51.1	46.4	68.6
11, Plantations	26.8	6.3	4.3	11.0	1.3	6.1
Total forest	1,602.4	25.7	562.0	49.8	230.5	48.3
Non-forest	889.2	43.2	426.6	53.2	153.3	54.5
Total PSF	1,366.8	27.6	452.3	57.2	200.1	55.5
Total peatland	2,153.3	33.9	854.8	55.5	337.6	54.7
Non-peatland	338.3	19.9	133.8	24.7	46.2	22.4
Total area	2,491.6	32.0	988.6	51.3	383.8	50.8

MRP, Mega Rice Project; PSF, peat swamp forest.

*Initial numbers represent classes: see text.

residual haze after the fires and by frequent cloud cover in the aftermath of the El Niño, so we used multitemporal, synthetic aperture radar (SAR) in addition to Landsat TM/ETM imagery to determine the extent of the burnt areas. The combined evaluation shows that 32.0% (796,907 ha) of the investigation area was burned, 91.5% (729,500 ha) of which was peatland. Of this fire-damaged peatland area, 47.4% (377,814 ha) was PSF (pristine, logged and fragmented) and the rest was degraded and deforested peatland (Fig. 1c). Only 4.5% of the pristine PSF was lost, while 29.2% of logged over and 70.0% of fragmented PSF were destroyed by fire. Severe damage also occurred to forest mosaics (54.1%), bushland (45.2%) and agricultural land (36.9%). The fire-damaged peatland represents 29.3% of the study area and 33.9% of the peatland within it.

We also compared the areas of each land cover type burned within and outside the boundary of the MRP in order to determine the effect of a major land use change on the incidence of fire. The most severely fire-damaged land cover types within the MRP were logged over and fragmented PSF and forest mosaics, of which 56.8%, 73.9% and 54.1% burned, respectively (Table 1). By the start of the fires in 1997, only a small area of pristine PSF remained within the MRP area and 14.1% of this burned, compared with only 3.6% of the much larger area of PSF outside the MRP. Clearly, forest clearance and peat drainage increased the likelihood of fire, because 51.3% of the MRP area burned compared to only 19.3% outside the MRP. This is confirmed by the spatial pattern of fire occurrences

(NOAA AVHRR hotspots in Fig. 1a; see methods) and burn scars within the MRP. These show that most damage occurred adjacent to the recently excavated drainage and irrigation channels along which people gained access (Fig. 1a, c). In comparison, the large area of relatively undisturbed, undrained PSF, outside the MRP to the west of the River Sebangau (to the left in Fig. 1a, c), had only a few scattered fire scars, probably associated with illegal logging.

To obtain more accurate information on the amount of carbon stored in this peatland landscape, and to determine how much of this was burned away in the fires, we carried out a detailed study of peat thickness and surface topography within Block C (383,800 ha), the most westerly part of the MRP between the Kahayan and Sebangau rivers (Fig. 1a). We found that this peat-covered landscape is dome-shaped, with a maximum thickness of 8 m. Analysis of 126 field drillings gave a mean peat thickness in Block C of 4.4 ± 0.2 m, equating to a mean volume of peat of $(14.9 \pm 0.3) \times 10^9$ m³. In order to compensate for those areas where the peat was shallow or non-existent (that is, <0.5 m surface organic layer; for example, close to the major rivers and near to the coast), we applied a Geographical Information System (GIS) inverse distance related (IDW) interpolation model to this entire interfluvial peatland catchment to ensure we did not over-estimate the mean peat thickness. This provided a reduced estimate of 2.3 m for average peat thickness throughout Block C, with a mean volume of 7.8×10^9 m³. It is very difficult to determine the amount of peat destroyed by the fires, because few measurements were made of this

Table 2 Effects of the 1997 fires on peat and carbon stores

	Block C of MRP, 383,800 ha	Entire MRP, 988,568 ha	Study area, 2,491,619 ha	Extrapolated values for whole of Indonesia, 190,400,000 ha		
				Lower estimate	Intermediate estimate	Upper estimate
Peatland area (ha)	337,632	854,809	2,153,304	20,072,825 (ref. 4)		
Peat volume* (10 ⁹ m ³)	7.8–14.9	19.7–37.6	49.5–94.7	461.7–883.2		
Carbon store*, † (Gt)	0.44–0.85	1.12–2.14	2.82–5.40	26.32–50.34		
Area of fire-damaged peatland (ha)	184,564 (48.1%)	474,009 (48.0%)	729,500 (29.3%)	1,450,000*	2,441,000 [†]	6,804,688 [‡]
Per cent of peatland area damaged	54.7%	55.5%	33.9%	7.2%	12.1%	33.9% [§]
Peat volume loss‡ (10 ⁹ m ³)	0.85–1.03	2.18–2.66	3.36–4.09	6.67–8.12	11.23–13.67	31.30–38.30
Peat carbon loss to atmosphere‡ (Gt)	0.05–0.06	0.12–0.15	0.19–0.23	0.38–0.46	0.64–0.78	1.78–2.17
Per cent peat carbon loss from store‡	5.9–13.6%	5.6–13.4%	3.5–8.2%	0.8–1.8%	1.3–3.0%	3.5–8.2%
Biomass carbon loss* (Gt)	0.01	0.03	0.05	0.10	0.17	0.40
Total carbon loss (Gt)	0.06–0.07	0.15–0.18	0.24–0.28	0.48–0.56	0.81–0.95	2.18–2.57

*Range derived from the IDW interpolation model, average peat thickness of 2.3 m; and 4.4 m from 126 field drillings.

†Based on a peat bulk density of 0.1 g cm⁻³, and peat carbon content of 57% (0.57)¹⁸.

‡Using 0.51 m for the estimated average depth by which peat burned down in the 1997 fires.

§Derived from the total area of peatland in Indonesia (20,072,825 ha; ref. 4) and an assumption, based on this study, that on 33.9% of this area an average of 0.51 ± 0.05 m of peat burned away.

||Based on the sum of: 730,000 ha for our 2.5-Mha study area, 700,000 ha for the rest of Central and West Kalimantan (derived from our own satellite imagery and land cover maps), 311,000 ha for East Kalimantan², 400,000 ha for West Papua³ and 300,000 ha for Sumatra². The data for West Papua are uncorroborated, and those for Sumatra are probably gross underestimates.

¶Ref. 9.

#Based on vegetation biomass¹⁵ carbon content of 250 t C ha⁻¹, 50% of which was liberated in the fires.

damage at the time and the evidence has now been covered over by regenerating vegetation. We obtained some data, however, on the thickness of peat burned away by measuring from the former peat surface, determined by observing adjacent, unburnt PSF, to the post-fire level. These varied between 25 and 85 cm with an average of 51 ± 5 cm (95% confidence limit). We estimated the loss of carbon to the atmosphere as a result of fires burning in the peat within Block C, and then extrapolated the probable carbon losses to the atmosphere from the whole of the MRP area, our study area of 2.5 Mha and the total peatland area of Indonesia in 1997^{9,10,14} (Table 2).

According to our data, 0.19–0.23 Gt C (3.5–8.2%) of the total carbon stored in the 2.5-Mha study area was released in the 1997 fires while, in the MRP, 0.12–0.15 Gt C (5.6–13.4%) of the peat carbon was transferred to the atmosphere. These values do not take into account the amount of carbon lost in biomass burning but, on the basis of a carbon content for pristine PSF¹⁵ of 250 t C ha^{-1} , and taking into account that 50% of the PSF trees remained standing after fire, we estimate this to be 0.05 Gt for the 2.5-Mha study area. The biomass produced on bushland, agricultural and plantation areas, collectively, is negligible compared to that on the various forest cover types and has been ignored.

It is almost impossible to determine, with precision, the total area of landscape in Indonesia that burned in the 1997 fires, or how much of that was peatland, because data are few, conflicting and vary enormously. A report by the Indonesian Development Planning Agency, for example, provides a range from 0.16 to 9.50 Mha for the whole of Indonesia⁹, while studies^{10,14} using low-resolution satellite imagery suggest that 3–13 Mha were affected in Kalimantan and Sumatra alone. None of these studies identified the area of peatland that was affected. The estimate of 1.45 Mha of peatland burned in the whole of Indonesia during 1997⁹ includes data for Sumatra (300,000 ha), Kalimantan (750,000 ha) and West Papua (400,000 ha) and provides a value of 0.48–0.56 Gt for carbon loss. We believe this to be an underestimate, because of (1) the inaccuracy of the methods used to determine the areas of different land cover types burned, (2) the lack of detailed satellite and ground truth information to differentiate between areas of peatland versus non-peatland that burned, and (3) the use of data for forested peatland only, whereas agricultural land on peat is excluded.

By applying our values of an average of 0.51 ± 0.05 m of peat burned away over 33.9% of the peat-covered landscape of Central Kalimantan to the 20.07 Mha of peatland in Indonesia⁴, and assuming that 50% of above-ground biomass (at 250 t C ha^{-1}) was burned, we estimate that between 2.18 and 2.57 Gt of carbon could have been released to the atmosphere from these catastrophic fires. We also provide an intermediate scenario, based on a fire-damaged peatland area of 2.44 Mha (derived from a combination of verifiable and uncorroborated sources) of a release of 0.81–0.95 Gt C. This is also likely to be an underestimate, because the information for Sumatra has not been acquired with the same degree of stringency as that for Kalimantan, and there are no reliable data for West Papua. We conclude that the most likely amount of carbon released from burning of peat and its surface vegetation in Indonesia was between 0.81 and 2.57 Gt. This is 13–40% of the current global annual carbon emissions from the burning of fossil fuels (6.4 Gt C yr^{-1}), and would have been a major contributor to the sharp increase in atmospheric CO_2 concentrations detected in 1998—from the 1990–99 average of $3.2 \pm 0.1 \text{ Gt C yr}^{-1}$ to 6.0 Gt C yr^{-1} , the highest value recorded since direct measurements began in 1957¹.

The extensive fire damage caused in 1997 has accelerated changes already being caused in tropical peatlands by forest clearance and drainage. We found, by assessing logging activity in Landsat images for our 2.5-Mha study area for 1997 and 2000, that logging had increased by 44% during this period, thus making the remaining forests more susceptible to fire in the future. This is a matter of

concern because natural, undamaged PSF is essential to maintain high water levels, protect the peat carbon store and facilitate future carbon sequestration from the atmosphere. If more PSF is destroyed by logging, development and fire, there will be a continued release of carbon through decomposition of the exposed peat surfaces that, in turn, will place this large carbon store at further risk. Tropical peatlands will make a significant contribution to global carbon emissions for some time to come unless major mitigation, restoration and rehabilitation programmes are undertaken. □

Methods

Satellite image processing and interpretation

Pre-fire land cover and the area burned were obtained from two Landsat TM images, one from shortly before the fires commenced in 1997 (Landsat TM 5, 118-62, 29 May 1997) and the other after the end of the fires (Landsat TM 5, 118-62, 29 March 1998). The two images were spectrally adjusted to each other by histogram matching, then contrast-enhanced, co-registered using a set of 24 ground control points, and geo-referenced using 36 Global Positioning System (GPS) measured ground control points. Interpretation of Landsat TM images (channels 1–5) was carried out by visual on-screen digitizing at a scale of 1:100,000 (minimal mapping unit 50 ha). The interpretation key for land cover and burned surfaces was established from GPS ground measurements. The land cover legend was adapted from the TREES classification scheme¹³. Logging activity was determined from two Landsat ETM images (118-62, 29 May 1997 and 12 August 2000) by visual image interpretation. Difference detection techniques¹⁶ to map the burned areas were applied to pairs of ERS SAR images acquired by the high resolution Active Microwave Instrument (AMI, 25-m ground resolution). This instrument detects structural features of the Earth's surface (volume scattering) and the moisture content of the vegetation (dielectric properties)¹⁷. Fire decreases both. Volume scattering decreases because fire consumes vegetation and moisture content decreases because fire-damaged plants lose their foliage. Furthermore, the opened canopy and the reduced leaf biomass allow more backscatter from the exposed ground surface. A mosaic of 6 ERS images was made out of 12 ERS images (frame nos 3645, 3663; orbits 07873, 11652, 12110, 12883, 13112, 32828) acquired before the fires (beginning of July 1997) and towards the end of the fire season (October 1997). Images acquired after rain had started again (November 1997) were not suitable for evaluation because surface water in swamps disturbed burn scar detection.

Pre- and post-fire images were co-registered to form bi-temporal image pairs. Co-registration was done automatically using the SAR Toolbox (ESA) with a registration error of less than one pixel. The images were calibrated to represent radar image backscatter, sub-sampled to 25 m pixel size, speckle filtered, and georeferenced (using orbital information and 36 GPS measured ground control points). The bi-temporal mosaic was visually interpreted on-screen (scale 1:200,000, minimal mapping unit 100 ha). The interpretation of SAR signatures was based on GPS-mapped ground observations and evaluation of 42 digital video sequences. Fire occurrence was confirmed using coarse resolution images from the Advanced Very High Resolution Radiometer (AVHRR, 1,100-m ground resolution) onboard the NOAA 12 and 14 satellites, which were processed to detect hot spots by the IFFM fire monitoring centre in Samarinda, East Kalimantan. During a period of 338 d from January 1997 to December 1997, 15,908 hot spots were recorded in the study area. An area was acknowledged as burned only when there was either clear decrease in backscatter, or weak decrease in backscatter in conjunction with NOAA-AVHRR hotspot evidence. Quantitative analysis and intersection of the resulting thematic maps were carried out in a Geographical Information System. The area was confined to the overlapping region in all remote sensing data sets ($25,000 \text{ km}^2$).

Ground surveys and field measurements

Since 1993, we have been carrying out large-scale ecological field research on PSF in Central Kalimantan, during which we have acquired an extensive database of information on tropical peatland biodiversity and natural resource functions. This ecosystem is almost inaccessible because it is flooded for up to 10 months of the year, the ground surface is very uneven, the vegetation is virtually impenetrable and there are several health and safety concerns. For this study, extensive ground-truthing, involving travel of over 3,000 km, had been carried out before the fires in order to check image classification of land-use and vegetation within the study area. Post-fire ground-truthing to verify the existence and magnitude of land cover types and burn scars was made at 145 GPS-recorded locations (Fig. 1b) and using 4 h of GPS synchronized video material acquired during two aerial surveys (conducted in 1998 and 1999) to check areas that could not be accessed on foot. Classification accuracy for discrimination of burned and unburned surfaces was >95%, based on 184 randomly selected digital video images.

Peat thickness was measured using a manually operated peat corer; we found that the PSF is very dense, and that trees are preserved within the peat mass. The surface levels of the peat-covered landscape relative to the adjacent main rivers, and the thickness of the underlying peat, were determined at 126 locations at intervals of 500 m along the main channels in Block C of the MRP. These channels were the only way to gain access to otherwise impenetrable burned and unburned PSF, and they enabled data to be collected on a grid basis at several locations both across and along the watershed. This peat inventory involved 6 people for a period of 4 months in 1999/2000. Peat volume was calculated from the peat area occupied by different land cover types, determined by satellite imagery, and GIS IDW interpolation of peat thickness, obtained from the field drilling. The IDW average was found to be adequate because all of the peatlands investigated in this study were dome-shaped and thus did not show extreme values. For the

IDW interpolation we set all boundary values to a minimum peat thickness of 50 cm. For the calculation we used cell size 100 m, nearest neighbour, power 2 and no barriers. For loss of peat thickness as a result of the fires, we made 43 measurements in several locations in mixed swamp forest in block C of the MRP, at a distance of about 7 km from the edge of the peat dome and more than 0.5 km from the nearest drainage channel.

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The origin of geomagnetic jerks

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Geomagnetic jerks, which in the second half of the twentieth century occurred in 1969 (refs 1, 2), 1978 (refs 3, 4), 1991 (ref. 5) and 1999 (ref. 6), are abrupt changes in the second time-derivative (secular acceleration) of the Earth's magnetic field. Jerks separate periods of almost steady secular acceleration, so that the first time-derivative (secular variation) appears as a series of straight-line segments separated by geomagnetic jerks. The fact that they represent a reorganization of the secular

variation implies that they are of internal origin (as has been established through spherical harmonic analysis⁷), and their short timescale implies that they are due to a change in the fluid flow at the surface of the Earth's core (as has also been established through mapping the time-varying flow at the core surface⁸). However, little is understood of their physical origin. Here we show that geomagnetic jerks can be explained by the combination of a steady flow and a simple time-varying, axisymmetric, equatorially symmetric, toroidal zonal flow. Such a flow is consistent with torsional oscillations in the Earth's core, which are simple oscillatory flows in the core that are expected on theoretical grounds⁹, and observed in both core flow models¹⁰ and numerical dynamo models¹¹.

In Fig. 1 we show the secular variation of the magnetic field at Niemegk (Germany) and Macquarie Island (Australia) observatories for the period 1950–2001. At Niemegk, the secular variation of the Y (east) component is simply described: it is linearly decreasing until around 1969, then linearly increasing until 1978, linearly decreasing to 1990, and linearly increasing to 1999. Although the abrupt nature of the geomagnetic jerks which mark the transitions between these intervals of nearly linear secular variation at 1969, 1978, 1991 and 1999 has been the focus of most attention, the near linear trend of the secular variation between the jerks is also noteworthy; for a review see ref. 12. However, at Macquarie Island (which is roughly antipodal to Niemegk) the secular variation is much less straightforwardly characterized, with little or no evidence of magnetic jerks.

The fact that jerks are most readily observed at European observatories, are largely confined to one component of the field, and are abrupt, argues for a local origin, perhaps a magnetic field instability. Arguing against such an origin is the fact that they represent transitions between long intervals of linear secular variation; in other words, jerks are not simply transient perturbations to the secular variation, instead they delineate intervals of oppositely signed secular acceleration.

The most powerful tool that we have available for investigating the origin of the secular variation is the frozen-flux hypothesis¹³, which states that on timescales short compared to that of magnetic diffusion the secular variation results primarily from the rearrangement of the magnetic field by fluid flow at the core surface. We find that a substantial portion of the secular variation over the past 150 years can be explained with a steady fluid flow¹⁴. Nonetheless, significant signal remains unexplained, including geomagnetic jerks. Here we explore whether the remaining signal can be explained with a simple, physically plausible, time-varying flow.

To a first approximation, the geomagnetic dynamo is expected to operate in a Taylor state⁹, where the magnetic field in the core is arranged so that the axial Lorentz torque vanishes when integrated over the surface of cylinders coaxial with the rotation axis. Small departures from this state would cause angular accelerations of these cylinders about the Earth's rotation axis, resulting in torsional oscillations, which are oscillatory differential rotations of these cylinders. Representing this flow at the core–mantle boundary using vector spherical harmonics¹⁵, we have

$$\mathbf{u}_T(s, t) = \nabla \times (T(s, t)\hat{\mathbf{r}}) \quad (1)$$

where $\mathbf{u}_T$ is the fluid flow, s is distance from the rotation axis, $\hat{\mathbf{r}}$ is a unit vector in the radial direction and t is time, with

$$T(s, t) = \sum_{l \text{ odd}} T_l(t) P_l^0(\cos \theta) \quad (2)$$

where θ is colatitude. This is an extremely parsimonious representation of the time-dependent part of the flow: for a flow up to spherical harmonic degree n , only $n/2$ out of the $2n^2 + 4n$ spherical harmonic coefficients of the flow are included. It is important to note that this work is distinguished from previous attempts to explain geomagnetic jerks with time-varying flow^{16–18} by the fact

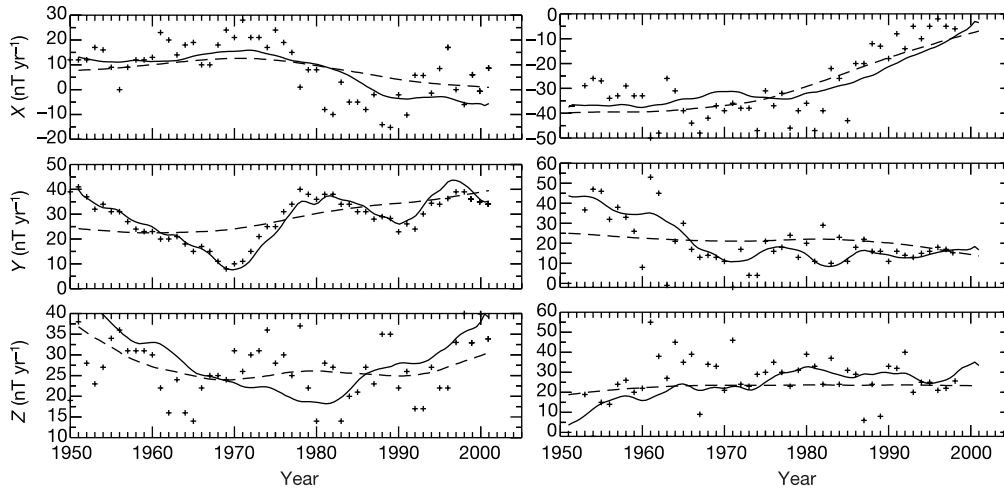


Figure 1 The secular variation (first derivative) of the magnetic field observed at Niemegk observatory (left; Germany) and at Macquarie Island observatory (right; south of Australasia). Crosses, differences between annual averages of the magnetic field; dashed lines, the secular variation predicted by the steady part of the flow at the surface of the core; and solid lines, the secular variation predicted by the steady part plus a time-dependent axisymmetric, equatorially symmetric zonal flow (the form of torsional oscillations) at the top of the core. Geomagnetic jerks can be clearly observed in the Y component of the field at Niemegk, and are well fitted by the time-dependent flow allowing

torsional oscillations, but not by the steady flow. In contrast, at Macquarie Island, which is approximately antipodal to Niemegk, there are no jerk-like features to be observed, and jerks are not predicted by either flow model. This suggests first that jerks can be explained by a simple model of core dynamics that includes torsional oscillations, and second that jerks are strongly dependent on the local magnetic field at the top of the core, and hence the fact that they are observed only in limited portions of the world does not imply that the underlying dynamics are also local.

that our choice of time-varying flow is based on core dynamics rather than on a mostly kinematic description of flow in the core.

We fit the flow $\mathbf{u} = \mathbf{u}_S(\theta, \phi) + \mathbf{u}_T(s, t)$ to a time-varying model of the magnetic field over the period AD 1950–2001 (which uses cubic B-splines with yearly knots to represent the time dependency), where $\mathbf{u}_S$ is a steady flow composed of both toroidal and poloidal harmonics and is dependent upon both colatitude, θ , and longitude, ϕ . This period includes four geomagnetic jerks. In Fig. 1 we show the fit of the flow $\mathbf{u}$ and its steady part ($\mathbf{u}_S$) to observatory data. The steady part of the flow reproduces the magnitude of the secular variation and its trend over this interval, but provides a poor fit to the Y component at Niemegk. The addition of the time-varying part of the flow improves the fit, most notably for the Y component at Niemegk. The basic features of the geomagnetic jerks at 1969, 1978, 1991 and, perhaps, 1999, are fitted. This simple time-varying flow is also able to reproduce some details of the jerks, such as the double peak observed around the 1978 jerk. The fact that this detailed structure can be explained with the addition of this simple time-varying flow, which is consistent with torsional oscillations in the core, argues that this short-period field variability is of internal origin.

However, the fit is not perfect, and is still smoother than the observations. There are two plausible explanations. First, the model of the magnetic field at the core–mantle boundary upon which this analysis is based is low-pass filtered by geometrical attenuation owing to the presence of the mantle and the effects of crustal fields. As a result, the secular variation generated by the time-varying flow may be incomplete, as the magnetic field model upon which it acts is incomplete, and the flow model itself is less well determined. We note that this may be a problem even though the magnetic field models fit the observatory data very well. Second, the time-varying flow may be more complicated, both spatially and temporally, than this simple symmetrical toroidal flow¹⁷. However, the important point to note is the improvement that this simple time-varying flow provides over the steady flow: clearly, a large part of the signal of geomagnetic jerks is explained by a parsimonious time-dependent flow.

Even though the additional components of the flow that we have added to obtain this fit are symmetrical about the geographical

Equator and symmetrical about the rotation axis (so that the additional flow beneath Niemegk at 52° N is almost identical to that beneath Macquarie Island at 54° S), jerks are neither observed at Macquarie Island nor predicted by the flow model. This demonstrates that the geographical distribution of geomagnetic jerks results from the morphology of the main magnetic field upon which the flow acts. In particular, we need not appeal to a localized phenomenon within the core to explain geomagnetic jerks: they can be adequately explained by global core dynamics.

The spatial parameterization of the time-varying flow was motivated by the form of torsional oscillations. Our next step, then, is to represent the time-varying flow as a sum of torsional oscillations—in other words, as a sum of waves of varying period and damping (or growth) timescale. Using a method akin to that of refs 10 and 19, we fit the time-varying part of the flow model (that is, the $T_l(t)$ coefficients) from 1957 (the International Geophysical Year, when there was a significant improvement in the global observatory coverage) to 2001 with a steady flow, a steadily accelerating flow, and three damped harmonic oscillations. The steadily accelerating component of the flow accounts for torsional oscillations with periods much longer than the timespan of the flow model used here, and for core dynamics on longer timescales than torsional oscillations. This model is illustrated in Fig. 2 (and see Table 1). The variance reduction is 99.965%, although much of this is due to the relatively large steady parts of the t_{odd}^0 coefficients: after subtracting the time average, the variance reduction is 98.4%. For comparison, the variance reduction from a fit with two damped harmonic oscillations after removing the time average part of the flow is 95.2%, and that from a fit with one damped harmonic oscillation is 87.4%.

The average power of the flow over the core's surface as a function of time is shown in Fig. 3 for the flow model and the fits with one, two and three waves. Although much of the power can be explained by the one-wave fit, the extra waves significantly improve the detail of the fit, particularly around the local minima and maxima that tend to be coincident with geomagnetic jerks.

Wave 1 may tentatively be identified with wave b of Zatman and Bloxham¹⁰ (which they found to be poorly resolved but for which they estimated a period of 53 years), whereas the other two waves are

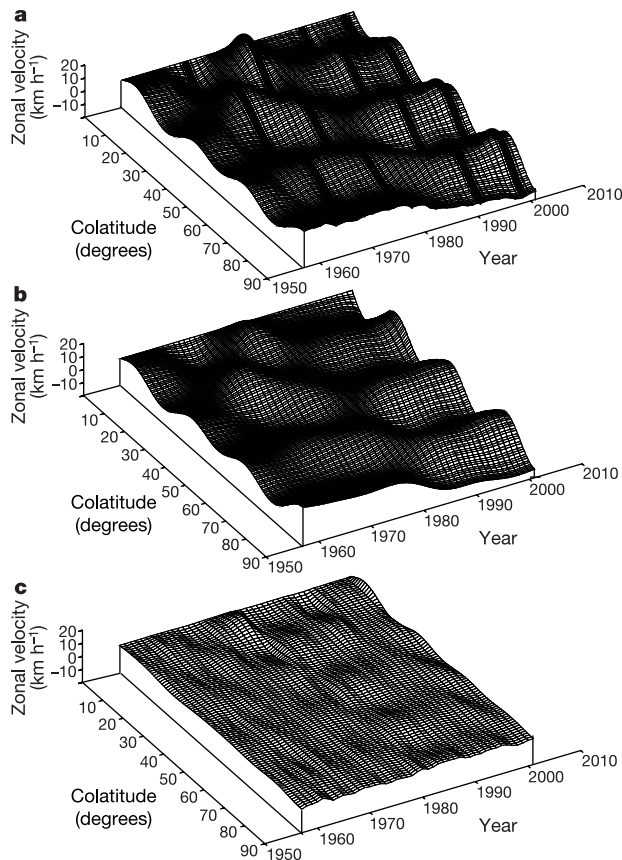


Figure 2 The time-dependent part of the flow model (after removing the time average) from 1957 to 2001. **a**, Time-dependent part of the flow model. The model is truncated at degree 20, although damping of spatial derivatives (together with weak damping of temporal derivatives) is applied to ensure convergence. The model is expanded in time by cubic B-splines with knots at one-year intervals. The flow at the times of geomagnetic jerks (1969, 1978, 1991, 1999) is picked out with bolder lines. **b**, Fit of a steadily accelerating flow plus three damped harmonic oscillations to the model shown in **a**. The fit performed is a least-squares fit to the T_l (l odd) spherical harmonic coefficients of the flow model from 1957 to 2001. **c**, Residuals.

faster than anything they were able to observe. However it is interesting to note that in contrast to the decaying waves of their study (over the epoch 1900–90), each of these waves is growing. This might be explained by increased excitation of torsional waves towards the end of the last century.

It is apparent that almost all of the flow can be fitted with a small number of oscillations, which firmly supports the idea that the observed signal can be understood in terms of (and hence is most probably due to) torsional oscillations.

We have presented a simple explanation of geomagnetic jerks, specifically that they result from torsional oscillations. Although torsional oscillations are expected in the core, the mechanism of their excitation is less certain, though, as suggested in ref. 9, they may arise simply as the means of relaxation of departures of the core from perfect magnetostrophic equilibrium. Such departures may

Table 1 Model parameters

Wave	Period	e^{-1} growth timescale
	(yr)	(yr)
1	45	22
2	20	55
3	13	46

See text for model details.

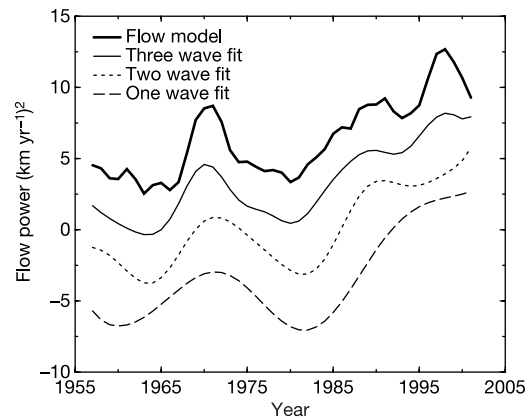


Figure 3 The spatially averaged power of the flow model from 1957 to 2001, compared with the one-, two- and three-wave fits to the flow model. (The three fits have been translated downwards by 9, 6 and 3 units, respectively, to improve visibility.) Although the one-wave fit to the flow model can account for much of its variance, the additional waves significantly improve the detail, especially around the local peaks and troughs that seem to be correlated with geomagnetic jerks.

themselves result from interactions of the convective flow with the magnetic field.

The present work suggests several possibilities for future investigation. Jerks may be a temporary phenomenon. This is because whether they occur in observatory records or not is a function of the morphology of the magnetic field rather than the underlying dynamical process, so with a different field morphology it is possible that they will not be observed anywhere. This may also explain why four geomagnetic jerks have occurred in the past 30 years whereas they were more scarce earlier in the last century. With other field morphologies jerks might, for example, not be observed in Europe, but instead be observed in the region antipodal to Europe. This work also extends studies of the secular variation to shorter periods: the double peak around 1978 (which is reproduced by the time-varying flow) is of short period, but, for the reasons we have given, probably of internal origin. If this double peak is indeed of internal origin, the fact that it is observed places a bound on the electrical conductance of the mantle. □

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Adaptive visual metamorphosis in a deep-sea hydrothermal vent crab

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Hydrothermal vents along the mid-ocean ridges host ephemeral ecosystems of diverse endemic fauna including several crustacean species^{1–4}, some of which undergo planktonic development as larvae up to 1,000 m above and 100 km away from the vents^{5,6}. Little is known about the role of vision in the life history of vent fauna. Here we report that planktonic zoea larvae of the vent crab *Bythograea thermydron* possess image-forming compound eyes with a visual pigment sensitive to the blue light of mesopelagic waters. As they metamorphose and begin to descend to and settle at the vents, they lose their image-forming optics and develop high-sensitivity naked-retina eyes. The spectral absorbance of the visual pigment in these eyes shifts towards longer wavelengths from larva to postlarva to adult. This progressive visual metamorphosis trades imaging for increased sensitivity, and changes spectral sensitivity from the blue wavelengths of the larval environment towards the dim, longer wavelengths⁷ produced in the deeper bathypelagic vent environment of the adults. As hydrothermal vents produce light⁷, vision may supplement thermal and chemical senses to orient postlarval settlement at vent sites.

Populating sparsely distributed hydrothermal vents presents challenges of dispersal and settlement for vent larvae, especially for those developing in the deep-sea plankton 100 km or more from their vent of origin^{5,6}. Hydrothermal activity gives rise to characteristic chemical signatures (for example, sulphide and methane gradients), which vent crustaceans may use to locate active hydrothermal fields⁸, as well as thermal gradients (2 °C to greater than 360 °C), which may be exploited once in the vent near-field. Light generated at the vents is sufficient to support vision^{9–11} in an otherwise aphotic world⁷ and could serve as a redundant near-field settlement cue if juveniles approaching the vents can detect it. We used the predatory Pacific vent crab *Bythograea thermydron* Williams (Decapoda: Brachyura: Bythograeidae) to examine the visual metamorphoses that accompany the transitions from the planktonic zoea larva to the postlarval megalopa to the first and third juvenile vent crab stages, and finally to the adult. Specimens

were collected from the East Pacific Rise and Galapagos Rift using the submersible DSV *Alvin*.

Zoea larvae of *B. thermydron* have a pair of apposition compound eyes positioned dorsolaterally on the anterior carapace (Fig. 1a). Each corneal lens is underlain by four corneagenous cells and a quadripartite crystalline cone (Fig. 1b). This dioptric apparatus focuses light onto a long, thin cylindrical rhabdom composed of microvilli from seven reticular (photoreceptor) cells in each ommatidium (Fig. 1b). Rhabdoms are ensheathed by screening pigment granules that absorb stray photons not absorbed by the rhabdoms (Fig. 1c). Similar apposition eyes are used by shallow-water marine crustacean larvae to maintain orientation and vertical position in the water column, avoid predators and select appropriate habitats¹². The photopigment in *B. thermydron* zoeal rhabdoms is rhodopsin-like with a maximum absorbance in the blue region of the visible spectrum ($\lambda_{\max} = 447$ nm) (Fig. 2). Transition from the larva to the megalopa includes a shift in spectral absorbance such that the megalopal photoreceptors are maximally sensitive to blue-green light ($\lambda_{\max} = 479$ nm) (Fig. 2). It is unclear whether this absorption is produced by a visual pigment unique to this developmental stage, or by a mixture of larval and adult visual pigments.

During metamorphosis from the megalopa to the first juvenile crab stage (stage 1; ref. 13) the entire image-forming dioptric apparatus is sacrificed to form a high-sensitivity naked-retina eye (Fig. 1d; see also ref. 14). Light enters the new eye through a transparent, non-faceted cornea; it passes through a thin corneal epidermis, and is absorbed by hypertrophied cylindrical rhabdoms that are approximately nine times larger in diameter than zoeal rhabdoms (Fig. 1e). Little or no screening pigment was observed in the new retina. Rhabdoms in ommatidia of the first juvenile crab stage are composed of long, thin, well-organized microvilli contributed by neighbouring reticular cells in orthogonal planes (Fig. 1f), which may provide the ability to analyse polarized light. As crustacean rhabdoms act as light guides with an acceptance angle of approximately 24° independent of optics¹⁴, the convex naked retina may derive some spatial information from the first juvenile crab stage's environment.

The naked-retina eye persists through subsequent moults to the third juvenile crab stage (stage 3; ref. 13) (Fig. 1g). Light now enters the eye through a cornea-like transparent layer of chitin and a thin unpigmented epidermis before reaching the retina (Fig. 1h). Densely packed rhabdoms appear contiguous in the outer retina to form a wall of visual-pigment-bearing membrane for photon absorption over a wide acceptance angle (Fig. 1h). The retina is similar to those of late-stage juvenile and adult bresiliid shrimps from vents on the Mid-Atlantic Ridge^{10,11,15}. In contrast to vent shrimp photoreceptors, which have well-organized microvillar rhabdoms, photoreceptors in the eye of the third juvenile crab stage have degenerate rhabdoms lacking microvilli (Fig. 1i). Degeneration of the rhabdoms may be an artefact of poor preservation of the rhabdom like that observed in the first description of the dorsal eye of the vent shrimp *Rimicaris exoculata* (compare refs 9 and 10). Alternatively, settlement at the vents may include a progressive increase in the susceptibility of the retina to light damage, rendering the rhabdoms sensitive to destruction by the operating lights of DSV *Alvin*. Submersible-induced light damage in the eyes of vent shrimps has been suggested^{10,16}.

Unlike the compound eyes of adult shallow-water brachyuran relatives, the eyes of adult *B. thermydron* (Fig. 1j) collected from the vents lack both a dioptric apparatus and anatomical evidence of a retina (Fig. 1k). The rhabdoms that are so prominent in the first and third juvenile stage crab eyes are missing in the adult eye. Instead, membrane-bound assemblages of an amorphous substance that binds little basophilic stain, and thus has low phospholipid content, fill the space behind the transparent cornea covering the eye. Photoreceptor somata, each a thin sheath of cytoplasm surrounding a nucleus, persist in the adult eye. The adult eye contains a visual

pigment with a peak spectral sensitivity ($\lambda_{\max} = 489 \text{ nm}$; Fig. 2) that is red-shifted relative to the photopigments of earlier stages, and can be photoconverted to a photostable metarhodopsin ($\lambda_{\max} = 460 \text{ nm}$).

This represents, to our knowledge, the first demonstration of successive metamorphosis of eye anatomy and photopigment content in adaptation to the demands of transition from a larval habitat in the deep-sea plankton to the bathypelagic vent environment. Eyes of zoea larvae contain a short-wavelength photopigment that is well matched to the dim blue light of mesopelagic (disphotic) waters. Descent towards the depths at which the vents occur along the East Pacific Rise where *B. thermydron* settles ($\sim 2,500 \text{ m}$) includes entry into the aphotic zone where solar photon fluxes at visible wavelengths are insufficient to support vision¹⁴. Hence, vision in the megalopa, most sensitive in the blue-green wavelengths, is potentially an adaptation to detect bioluminescence¹⁷ in deeper bathypelagic waters. Near the bottom, hydrothermal vents generate light in the near-infrared⁷ and visible¹⁸ portions of the spectrum. Sulphide oxidation at the vents, for example, may generate chemi-

luminescence at visible wavelengths between 380 and 620 nm (ref. 19). Crystalloluminescence, triboluminescence and sonoluminescence in the vent effluent probably contribute to the visible light¹⁸. The naked-retina eyes of the first juvenile crab stage of *B. thermydron* are well suited to serve as photon-gradient detectors, supplementing chemical and thermal senses for proper orientation towards the vents during settlement and assisting in the selection of an appropriate habitat for juvenile life. Eyes of third-stage juvenile crabs are similarly adapted to detect dim light and may be even more sensitive than the eyes of the first-stage juvenile crabs given their dense rhabdom packing and apparent lability to light and/or fixation. Negative phototaxis to the vent light in settled crabs would prevent them from being scalded while foraging dangerously close to localized venting⁹.

Adult *B. thermydron* may live for more than 10 yr with an adult intermoult duration of 3–4 yr (ref. 20). Our inability to detect structural evidence of a retina in adult crabs may be a result of chronic exposure to artificial lighting from repeated submersible visits, resulting in retinal degeneration. However, the presence of a

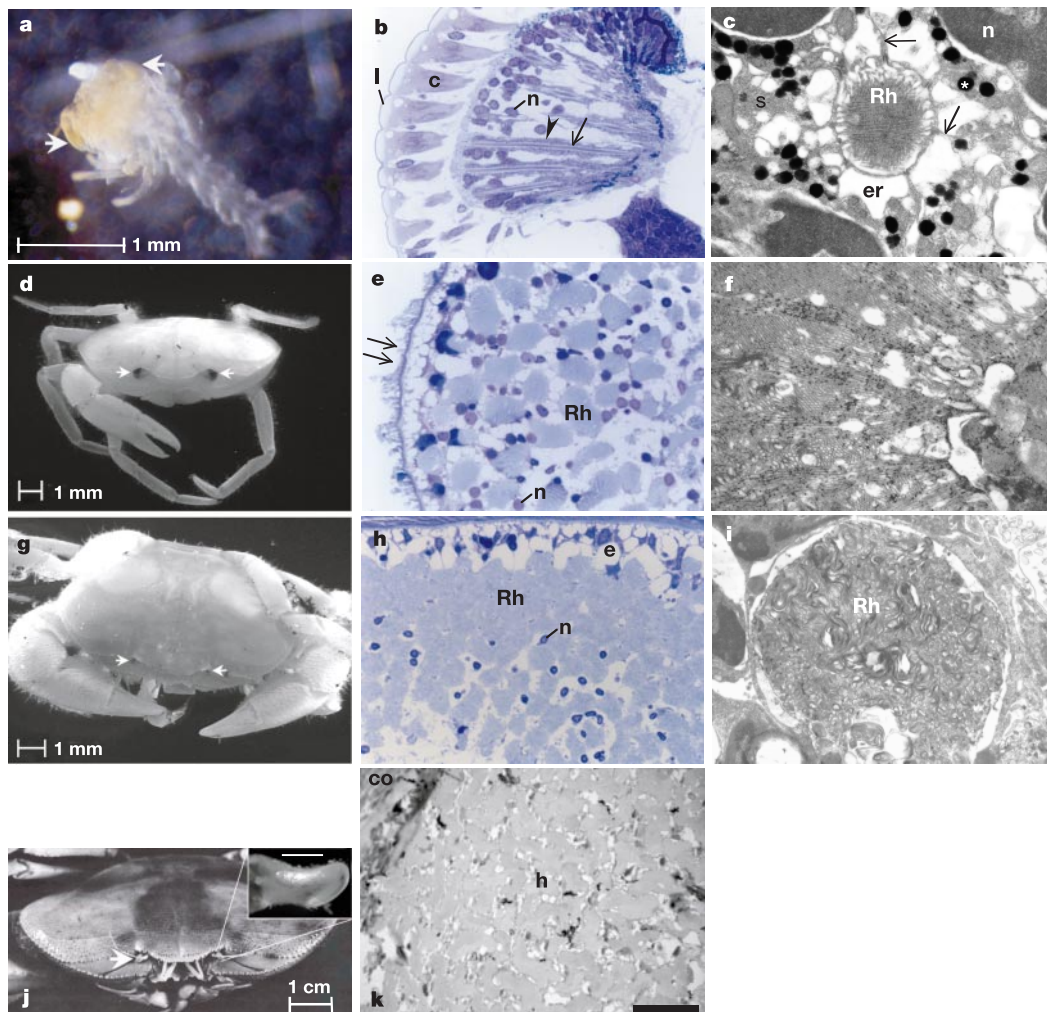


Figure 1 Ontogeny of the eye of *B. thermydron*. **a**, Zoea with apposition compound eyes (arrows). **b**, Longitudinal section of a zoeal eye displaying corneal lenses (l), crystalline cones (c) and photoreceptor cells (arrowhead) with cylindrical rhabdoms (arrow). **c**, Electron micrograph of a cross-section through the rhabdom (Rh). The rhabdom is surrounded by endoplasmic reticulum (er) with cytoplasmic bridges (arrows) linking it to the photoreceptor cell somata (s). **d**, First juvenile crab stage with reduced stalk naked-retina eyes (arrows). **e**, Cross-section through the hypertrophied rhabdoms (Rh) of the eye of the first juvenile crab stage. The smooth cornea separated from the retina during sectioning (arrows). **f**, First juvenile crab stage rhabdom displaying orthogonal layering of

microvilli. **g**, Third juvenile crab stage with reduced stalk naked-retina eyes (arrows). **h**, A smooth cornea (top) and thin epidermis (e) overlie contiguous rhabdoms (Rh) in the eye of the third juvenile crab stage. **i**, Cross-section through the rhabdom (Rh) of an ommatidium in the inner retina (lower half of **h**). **j**, Adult (adapted from ref. 23 with permission) with eyes on reduced stalks (arrow and inset). Bar in inset, 1 mm. **k**, Adult eye: a smooth cornea (co) is underlain by aggregations of an amorphous substance (h) where the rhabdom used to be in the juvenile retinas. Symbols used throughout Fig. 1: n, photoreceptor cell nucleus; asterisk, screening pigment granule. Bar in **k** represents approximately 35 μm in **b**, **e**, **h**, **k** and approximately 1.5 μm in **c**, **f**, **i**.

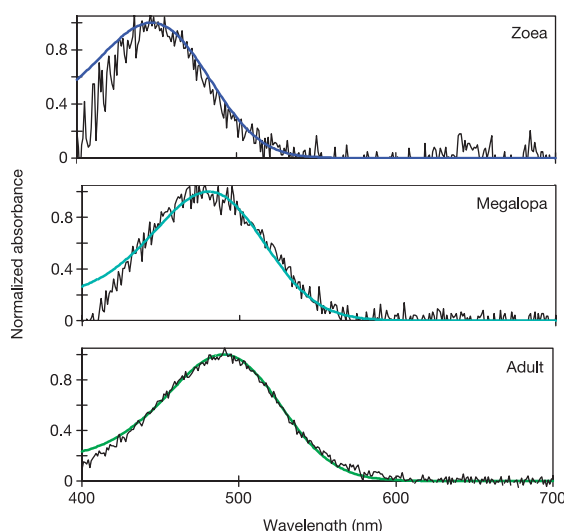


Figure 2 Absorbance spectra for *B. thermydron* visual pigments. Coloured curves are Stavenga-type rhodopsin templates²². Top, the zoeal visual pigment is fitted best by a template with $\lambda_{\max} = 447$ nm ($n = 6$ scans). Middle, the megalopa, the last instar before the juvenile crab, possesses a visual pigment with $\lambda_{\max} = 479$ nm ($n = 3$ scans). Bottom, adult eyes contain a red-shifted rhodopsin-like pigment with $\lambda_{\max} = 489$ nm ($n = 18$ scans).

rhodopsin-like photopigment in adult eyes preserved by rapid freezing suggests that the rhabdoms of *B. thermydron* beyond the first juvenile crab stage may be resistant to the chemical fixation used for anatomical analysis. Rapid freezing of crabs collected remotely under low light conditions at infrequently visited vents may be required to determine the *in situ* state of adult and late-juvenile eyes.

Because related shallow-water brachyuran crabs do not undergo visual metamorphosis^{12,21}, the metamorphosis of the eyes of *B. thermydron* described here appears to be a specific adaptation for life at the vents. □

Methods

Animals

Specimens were collected live under artificial illumination from DSV *Alvin*. Megalopae, collected at the vents, were identified by morphological and genetic analysis as described elsewhere¹³. Zoea larvae were hatched in darkness on board ship from a freshly collected ovigerous *B. thermydron*. Larvae were transported back to land in darkness, sorted briefly under ambient lighting, and returned to darkness before use.

Microscopy

Upon *Alvin*'s return to the surface (under natural light), whole juvenile crabs and excised adult eyes were submersed in fixative (5% paraformaldehyde w/v, 0.8% glutaraldehyde v/v, 4.5% sucrose w/v, 3% NaCl w/v in 0.1 M phosphate buffer; pH 7.2) under ambient illumination aboard RV *Atlantis*. Larvae were fixed by immersion under ambient lighting in Lancaster, Pennsylvania, 17 d after hatching. Adults and juveniles were stored in fixative at 4 °C in darkness until they were processed for microscopy on land by using standard methods¹⁰. Sample sizes: zoea ($n = 7$ eyes from 7 animals); juvenile crab stage 1 ($n = 6$ eyes from 3 animals); juvenile crab stage 3 ($n = 4$ eyes from 2 animals); adult ($n = 4$ eyes from 2 animals).

Microspectrophotometry

Animals were kept in darkness for several days before being used. Adult eyes were dissected out, quick-frozen using cryogenic spray, and sectioned at 14 μ m. Larvae and juveniles were frozen whole, but otherwise treated like the adults. Sections were collected on coverslips, mounted in marine crustacean Ringer solution containing 2.5% glutaraldehyde v/v (to enhance photobleaching), and placed in the microspectrophotometer. Photoreceptors were selected for scanning in dim, red light, and scanned using a beam (1.5–5 μ m) placed in each rhabdom. Rhabdoms were scanned twice: first when fully dark-adapted, and subsequently after being photobleached for several minutes with bright, white light. The difference between the scans was taken to be the spectrum of the visual pigment. Between 3 and 32 individual rhabdoms (depending on the developmental stage under study and the quality of the material) were scanned, bleached, and averaged for spectral characterization. Averaged scans were fitted mathematically with Stavenga templates²², using a least-squares procedure, to determine their characteristic wavelengths of maximum absorption.

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Neurons in medial prefrontal cortex signal memory for fear extinction

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Conditioned fear responses to a tone previously paired with a shock diminish if the tone is repeatedly presented without the shock, a process known as extinction. Since Pavlov¹ it has been hypothesized that extinction does not erase conditioning, but forms a new memory. Destruction of the ventral medial prefrontal cortex, which consists of infralimbic and prelimbic cortices, blocks recall of fear extinction^{2,3}, indicating that medial prefrontal cortex might store long-term extinction memory.

Here we show that infralimbic neurons recorded during fear conditioning and extinction fire to the tone only when rats are recalling extinction on the following day. Rats that froze the least showed the greatest increase in infralimbic tone responses. We also show that conditioned tones paired with brief electrical stimulation of infralimbic cortex elicit low freezing in rats that had not been extinguished. Thus, stimulation resembling extinction-induced infralimbic tone responses is able to simulate extinction memory. We suggest that consolidation of extinction learning potentiates infralimbic activity, which inhibits fear during subsequent encounters with fear stimuli.

Rats were given auditory fear conditioning in a 2-day experiment^{2,4}. The conditioned stimulus was a 30-s tone, and the unconditioned stimulus was a 0.5-s foot-shock that terminated simultaneously with the tone. On day 1, rats received five tones (habituation phase) followed immediately by five tones paired with foot-shock (conditioning phase). After 1 h, rats received 20 tones without foot-shock (extinction phase). On day 2, an additional 15 tones were given to test for recall of extinction learning. Freezing to the tone increased to 75% during the conditioning phase, and decreased to 10% by the end of the extinction phase (Fig. 1b). Twenty-four hours later, rats showed 35% freezing, which reflects good recall of extinction learning compared with the 80% freezing in animals that were not extinguished (data shown in Fig. 4b).

A total of 74 neurons were recorded from the medial prefrontal cortex (mPFC) across the 2 days. Cells were located in the infralimbic cortex (IL, $n = 31$, see Fig. 1a), prelimbic cortex (PL, $n = 25$) or medial orbital cortex (MO, $n = 18$). The initial spontaneous firing rate of all cells was 4.6, 5.5 and 4.2 Hz for IL, PL and MO, respectively. Spontaneous rates did not change significantly across the different phases of the experiment ($P > 0.3$, one-way analyses of

variance (ANOVAs) with repeated measures). We therefore focused on the tone-elicited activity of neurons. Figure 1c shows an example of a tone-responsive neuron in IL. No tone-elicited activity was observed during habituation, and cells remained unresponsive to tones during the conditioning phase. This agrees with previous studies showing that lesions of mPFC do not prevent acquisition of fear conditioning^{2,3,5}. Thus, unlike more dorsal parts of mPFC⁶, IL does not signal the acquisition phase of fear conditioning.

IL cells continued to be unresponsive to tones during extinction training on day 1. By day 2, however, robust tone-elicited activity in IL was visible from the start of the extinction phase. The increase in the neuronal tone response from day 1 to day 2 was inversely correlated with the spontaneous recovery of freezing on day 2 ($r = -0.73$, $P < 0.01$; Fig. 2a). Rats with the largest increase in IL tone responses showed the least freezing. To investigate further the relationship between IL activity and freezing, we divided the rats into two groups: those showing less than 50% spontaneous recovery of freezing and those showing more than 50% spontaneous recovery. As shown in Fig. 2c, IL activity was significantly increased 100–400 ms after tone onset in low-recovery rats but not in high-recovery rats. IL tone responses on day 2 are unlikely to reflect recall of conditioning because they were lowest in animals that showed the highest freezing. A more parsimonious explanation is that IL tone responses reflect extinction memory. Extinction-induced tone responses were not observed in nearby PL or MO (see Fig. 2c), indicating a high degree of anatomical specificity in the ability of mPFC to signal extinction memory.

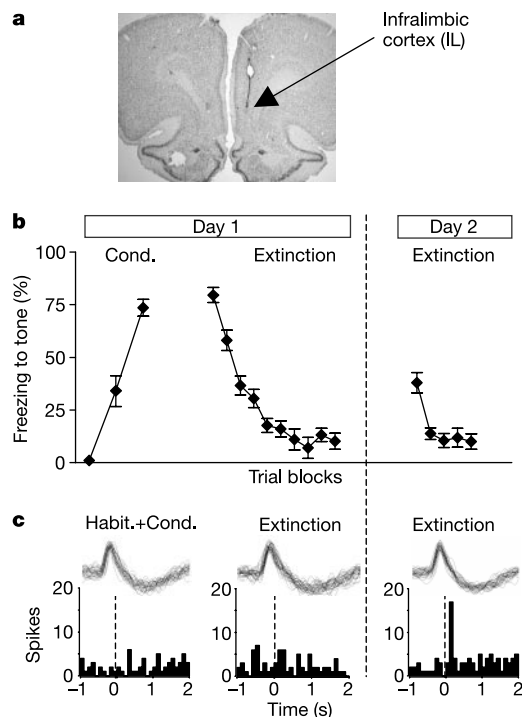


Figure 1 Tone response of a representative neuron in medial prefrontal cortex (infralimbic area, IL) during acquisition and extinction of conditioned fear. **a**, Unit-recording electrode in IL. **b**, Freezing to the tone shown in blocks of two trials for 24 rats. Freezing was low on day 2, indicating good recall of extinction learning. **c**, Waveforms and post-stimulus time histograms (PSTHs) showing the tone-elicited activity of a representative IL neuron in each phase of the experiment (10 trials each, bin = 100 ms). Dashed line indicates tone onset. IL neurons only signalled the tone 24 h after extinction training. Cond, conditioning; habit, habituation.

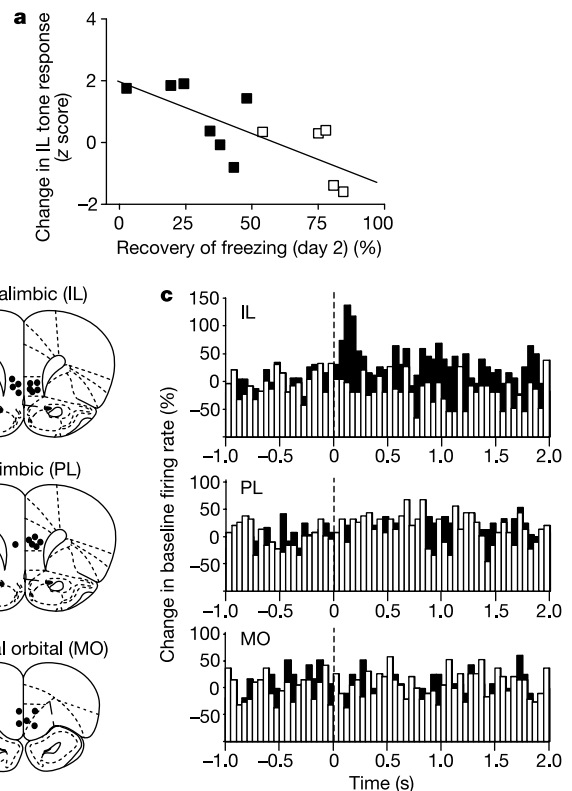


Figure 2 IL tone responses are correlated with spontaneous recovery of freezing after extinction. **a**, Scatter plot showing the change in IL tone response across days versus the percentage recovery of freezing on day 2. Firing rate 0–400 ms after tone onset was compared to pre-tone baseline rate with z-score. Each point represents the averaged response of all recorded neurons in each rat. Filled squares, low-recovery group (<50% 7 rats, 19 cells); open squares, high-recovery group (>50% 5 rats, 12 cells). $r = -0.73$; $P < 0.01$. **b**, Recording sites in IL, PL and MO. **c**, Group PSTHs showing tone responses of neurons from high-recovery (IL, 12 cells; PL, 12 cells; MO, 9 cells) and low-recovery (IL, 19 cells; PL, 13 cells; MO, 9 cells) groups on day 2. The bin size was 50 ms.

Figure 3 shows the average tone response of all IL neurons in each phase of the experiment. The difference in tone-elicited IL activity between high-recovery and low-recovery groups was significant on day 2 (Student's *t*-test, $t = 3.88$, degrees of freedom (d.f.) = 29, $P < 0.01$, Bonferroni corrected). On day 1 there was no relationship between IL activity and freezing. Cells in both groups were unresponsive to the tones despite large increases and decreases in freezing. This pattern of IL activity bears a striking resemblance to the effect of mPFC lesions in this task² (but see ref. 7). Lesions of the ventral mPFC with more than 70% destruction of IL had no effect on conditioning or extinction on day 1. However, 24 hours later, lesioned rats acted as though they had never received extinction training, spontaneously recovering high levels of freezing². Collectively, these data suggest a post-training consolidation process for extinction in IL. If this process fails to occur, either naturally or because of a mPFC lesion, extinction is not recalled.

The correlation that we observed between IL activity and freezing on day 2 indicates that IL tone responses are responsible for the suppression of freezing after extinction. However, correlations do not imply causality. Enhanced IL tone responses could be an epiphenomenon or a purely cognitive process that is not reflected in behaviour⁸. To determine whether tone-elicited activity in IL actually decreases freezing, we electrically stimulated IL during tones on day 2 in rats that were conditioned but not extinguished on day 1, to simulate a post-extinction state. Tones were paired with low-intensity IL stimulation delivered 100–400 ms after tone onset to model IL tone responses (see Fig. 4a). Control groups received either IL stimulation unpaired with tones, or no stimulation.

As shown in Fig. 4b, rats that received tones paired with IL stimulation showed markedly less freezing than controls on day 2. This effect was evident from the very first trial. Freezing levels in trial 1 were 82%, 90% and 60% in unstimulated, unpaired-stimulated and paired-stimulated rats, respectively. One-way ANOVA revealed a significant main effect of group ($F_{(2,29)} = 7.09$, $P < 0.01$), and post-hoc analysis showed that the stimulated-paired group was significantly lower than both controls ($P < 0.05$). The significant decrease in freezing in trial 1 is consistent with the simulation of extinction memory by IL stimulation.

IL stimulation also accelerated extinction learning. Across all four

trial blocks, average freezing levels were 70%, 73% and 28%, in unstimulated, unpaired-stimulated and paired-stimulated rats, respectively. ANOVA showed a highly significant main effect of group ($F_{(2,29)} = 16.38$, $P < 0.001$) and trial block ($F_{(4,116)} = 25.62$, $P < 0.001$), as well as a significant interaction ($F_{(8,116)} = 3.06$, $P < 0.01$). Freezing in the paired-stimulated group was significantly lower than controls in all blocks ($P < 0.01$). In contrast to IL, paired stimulation of PL ($n = 10$) had no effect (PL, 76%; unstimulated, 70%; $t = 0.71$, d.f. = 17, $P > 0.40$). Thus, similar to our unit-recording findings, the effect of stimulation was specific to IL. On day 3, IL-stimulated rats continued to show low freezing to the tone in the absence of stimulation (post-hoc test: $P < 0.05$; see Fig. 4b), providing further evidence of facilitated extinction learning. Enhanced extinction learning could be mediated directly by stimulation (for example, inducing extinction-related plasticity in IL or downstream structures) or indirectly through decreased freezing. A decrease in freezing in early trials might accelerate extinction by removing the behavioural feedback that maintains freezing on subsequent trials. Either way, this raises the interesting possibility that increased IL activity during recall might serve to strengthen extinction memory.

IL stimulation did not decrease freezing through a non-specific increase in locomotion. Rats stimulated during the inter-tone interval (unpaired group) showed no decrease in freezing during subsequent tones, or during the 30-s period immediately after stimulation (post-stimulation, 33% freezing; pre-stimulation 31%; $t = 0.15$, d.f. = 22, $P > 0.80$). Another possible explanation for the decreased freezing during the tone is that IL stimulation was rewarding⁹. This is unlikely because rewarding stimulation would also be expected to decrease freezing during the inter-tone interval. To address this issue further, we trained a separate group of rats ($n = 5$) to press a bar for food and then replaced food reward with press-elicited stimulation of IL, using the same stimulus parameters as above. For food, rats pressed at a rate of $20 \pm 2.0 \text{ min}^{-1}$ (mean $\pm$ s.e.). Switching to IL stimulation caused the press rate to drop to $10 \pm 1.5 \text{ min}^{-1}$ after 1 day and $0.3 \pm 0.06 \text{ min}^{-1}$ after 7 days. Thus the stimulation used in our experiment did not sustain self-stimulation behaviour, arguing against a reward mechanism of action.

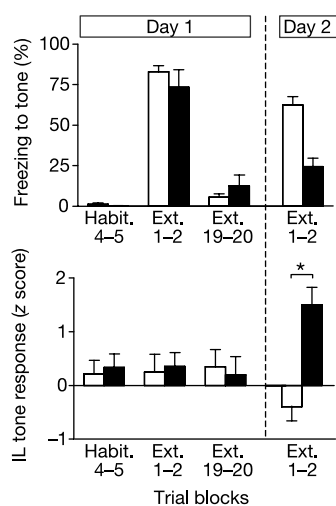


Figure 3 Average freezing (top) and IL tone response (bottom) in each phase of training. Numbers indicate trials. Despite increases and decreases in freezing during conditioning and extinction (ext.) on day 1, IL cells showed no tone responses. On day 2, however, neurons in low-recovery rats (filled squares) showed significantly larger tone responses than in high-recovery rats (open squares), indicating that IL neurons selectively signal recall of fear extinction. The asterisk indicates a statistically significant ($P < 0.01$) difference. Habit., habituation.

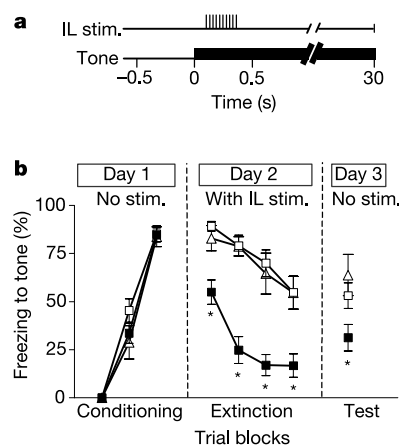


Figure 4 Brief IL stimulation (stim.) paired with tones simulates extinction memory. **a**, A train of pulses was delivered to IL 100–400 ms after tone onset on day 2 in non-extinguished rats, to model IL tone responses observed after extinction (see Fig. 2c). **b**, Percentage freezing to the tone for unstimulated ($n = 9$; open triangles), unpaired IL stimulated ($n = 11$; open squares) and paired IL stimulated ($n = 12$; filled squares) rats. The paired-stimulated group showed significantly lower freezing than controls at all points on day 2 ($P < 0.001$, asterisk). Paired-stimulated rats continued to show low freezing on day 3 ($P < 0.05$, asterisk) in the absence of stimulation. Data are shown in blocks of 2-trials.

Our observation that IL neurons are responsive to tones after extinction, but not during extinction itself, indicates that IL-dependent mechanisms are responsible for long-term, but not short-term, extinction memory. Separate extinction mechanisms are also indicated by pharmacological evidence: short-term memory for fear extinction seems to be independent of *N*-methyl-D-aspartate (NMDA) receptors^{4,10}, whereas long-term memory for extinction requires NMDA receptors^{4,11}. We suggest that post-training consolidation of extinction involves the potentiation of tone inputs to IL, perhaps by means of NMDA-dependent plasticity. The basolateral amygdala complex (BLA), which is crucial for the learning and expression of auditory fear conditioning^{12–14}, sends direct excitatory projections to IL^{15–17}. Microinjection of NMDA antagonists¹¹ or protein kinase inhibitors¹⁸ into BLA blocks the extinction of fear. Inputs to IL from BLA are therefore a likely candidate for potentiation during consolidation of extinction.

Once potentiated, how does IL influence freezing? The central nucleus of the amygdala modulates the expression of conditioned fear responses by means of projections to midbrain and hypothalamic sites that mediate fear responses¹⁹. IL sends robust projections to the capsular division of the central nucleus, which contains amygdaloid intercalated cells²⁰. Intercalated cells strongly inhibit output from the central nucleus²¹. When sufficiently depolarized, intercalated cells show sustained firing lasting tens of seconds in response to a brief suprathreshold stimulus²². This could account for the prolonged decrease in freezing that we observed with brief stimulation of IL. Thus, extinction-induced activation of IL neurons might decrease freezing by dampening the output of the amygdala. In support of this, prolonged stimulation of IL prevents increases in blood pressure and defensive behaviours elicited by amygdala stimulation²³.

Our data provide physiological evidence for the long-standing notion that extinction forms a new memory that inhibits the conditioned response^{1,24}. Failure to achieve an adequate level of potentiation in medial prefrontal cortex after extinction might lead to exaggerated fear responses²⁵. In support of this, patients with post-traumatic stress disorder exhibit depressed ventral mPFC activity correlated with increased autonomic arousal, when re-exposed to traumatic reminders^{26–28}. Pairing reminder stimuli with activation of ventral mPFC induced by repetitive transcranial magnetic stimulation²⁹ might serve to strengthen extinction of fear in a clinical setting. □

Methods

Subjects

Male Sprague–Dawley rats weighing 300–350 g were maintained on a restricted diet until they reached 85% of their original body weight. They were trained to press a bar for food on a variable-interval schedule of reinforcement (VI-90 s) to maintain a constant level of activity against which freezing responses could be reliably measured²⁴. Bar-pressing occurred in a standard operant chamber with dimensions 25 cm × 29 cm × 28 cm (Coulbourn Instruments).

Surgery and histology

The surgical procedures performed were similar to those previously described¹². Rats were anesthetized with Nembutal (50 mg kg⁻¹ intraperitoneally) after pretreatment with atropine (0.24 mg kg⁻¹ intraperitoneally). An eight-channel movable microelectrode was implanted in the ventral mPFC, with coordinates 2.9 mm anterior, 0.5 mm lateral and 3.9 mm ventral to bregma. Wires were arranged either individually or twisted in pairs to form four stereotrodes with a tip impedance of 1–3 MΩ. Rats were allowed to recover for 1 week before recording. At the conclusion of the experiment, recording sites were marked with electrolytic lesions before perfusion, and electrode locations were reconstructed with standard histological techniques.

Fear conditioning

Fear conditioning took place in the same operant chamber as bar-pressing. The chamber was located in a sound-attenuating box, and food reward was available continuously on a VI-90 s schedule throughout the experiment. The conditioned stimulus was a tone (4 kHz, 80 dB sound pressure level, 30 s), and the unconditioned stimulus was a scrambled foot-shock (0.5 mA, 0.5 s) that terminated simultaneously with the tone. The inter-trial interval varied from 2 to 6 minutes (average 4 min) throughout. The behaviour of rats was recorded with a video camera for offline scoring of freezing.

Single-unit recording

Electrodes were connected to a headstage (SUNY-HSC Biomedical Engineering Services) containing eight unity-gain operational amplifiers. The headstage was connected to an eight-channel, computer-controlled, preamplifier (Lynx-8) via a rotating commutator (Crist Instruments). The electrode was advanced in 40-μm steps until well-isolated cells were obtained in at least two or three wires, at which time fear conditioning began. Signals exceeding a voltage threshold were digitized at 32 kHz and stored on a PC (DataWave Technologies). The occurrences of tone and shock onset were flagged in the recording files. Waveforms were sorted off-line by using waveform characteristics such as peak amplitude, valley amplitude and spike width, and a clustering algorithm (Autocut; DataWave Technologies). Individual cells were tracked from day 1 to day 2 by applying cluster boundaries from day 1 and checking manually for goodness of fit. Waveforms were considered constant across the two days when they fitted the boundaries from day 1 and showed similar waveform shapes. Of 100 cells recorded, 26 did not fit these criteria and were rejected.

Brain stimulation

Rats were surgically implanted with concentric bipolar stimulating electrodes (Rhodes Medical Instruments) 0.2 mm in diameter with an exposed tip length of 0.25 mm. Stimulating electrodes were implanted into the right IL (2.7 mm anterior, 1.0 mm lateral, and 4.9 mm ventral to bregma, angled 6° toward the midline). Rats were implanted unilaterally rather than bilaterally to minimize track-related damage to the dorsal mPFC, which has been shown to increase conditioned freezing³⁰. On day 1, rats were conditioned as described above, but not extinguished. On day 2, rats received eight extinction tones paired with IL stimulation. A pulse generator with constant current output (Grass Instruments) delivered a 300-ms train of square pulses (0.2 ms pulse width, 100 μA, 100 Hz). Trains were delivered one per tone, in either paired (100–400 ms after tone onset) or unpaired (middle of inter-trial interval) fashion. An unstimulated control group was implanted with stimulating electrodes but was never stimulated. A separate group of rats implanted with stimulating electrodes in IL were trained to press for food on a variable-interval schedule of reinforcement (VI-60 s). Food reward was then replaced with stimulation of IL on a VI-15 s schedule. Rats were allowed to press for IL stimulation during daily 30-min sessions for 7 days, following a previously used protocol for studying intracranial self-stimulation in mPFC⁹.

Data analysis

Spontaneous recovery of freezing on day 2 was measured as the freezing at the start of day 2 (extinction trials 1 and 2) divided by the peak acquired freezing on day 1 (extinction trials 1 and 2). Low spontaneous recovery indicates good recall of extinction learning from the previous day. To measure tone-induced activity of neurons, the firing rate in the first 400 ms after tone onset was compared with the firing rate of 20 pre-tone bins of equal duration using a *z*-score transformation. The change in tone-induced activity of neurons across days was determined by subtracting the *z*-score at the end of the extinction phase on day 1 (trials 19 and 20) from the *z*-score at the beginning of the extinction phase on day 2 (trials 1 and 2). Group post-stimulus time histograms were generated by summing the spike count of all cells per bin and normalizing to the average pre-tone firing rate. ANOVA or Student's *t*-tests were used to compare neural tone responses (*z*-scores), firing rates and spontaneous recovery of freezing. Post-hoc comparisons were made with Tukey's HSD ('Honestly Significantly Different') method.

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p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp

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In inhibiting neurite outgrowth, several myelin components, including the extracellular domain of Nogo-A (Nogo-66)¹, oligodendrocyte myelin glycoprotein (OMgp)² and myelin-associated glycoprotein (MAG)^{3,4}, exert their effects through the same Nogo receptor (NgR). The glycosyl phosphatidylinositol (GPI)-anchored nature of NgR indicates the requirement for additional transmembrane protein(s) to transduce the inhibitory signals into the interior of responding neurons. Here, we demonstrate that p75, a transmembrane protein known to be a receptor for the neurotrophin family of growth factors^{5,6}, specifically interacts with NgR. p75 is required for NgR-mediated signalling, as neurons from p75 knockout mice are no longer responsive to

myelin and to each of the known NgR ligands. Blocking the p75–NgR interaction also reduces the activities of these inhibitors. Moreover, a truncated p75 protein lacking the intracellular domain, when overexpressed in primary neurons, attenuates the same set of inhibitory activities, suggesting that p75 is a signal transducer of the NgR–p75 receptor complex. Thus, interfering with p75 and its downstream signalling pathways may allow lesioned axons to overcome most of the inhibitory activities associated with central nervous system myelin.

A recent study suggested that p75 does not interact directly with MAG, but is required for its activity in inhibiting neurite outgrowth⁷. As NgR is a functional receptor of myelin-associated inhibitors including MAG^{1–4}, we examined the possibility that p75 and NgR formed a receptor complex in mediating these inhibitory activities. We first overexpressed haemagglutinin (HA)-tagged rat full-length p75 in both Chinese hamster ovary (CHO) cells (data not shown) and CHO cells stably expressing Flag-tagged human NgR, and found that p75 could be immunoprecipitated together with NgR, but not with a control transmembrane protein plexin A3 (ref. 8; Fig. 1a). Similarly, endogenous p75 and NgR proteins in rat postnatal cerebellar granule neurons (CGNs) could be immuno-

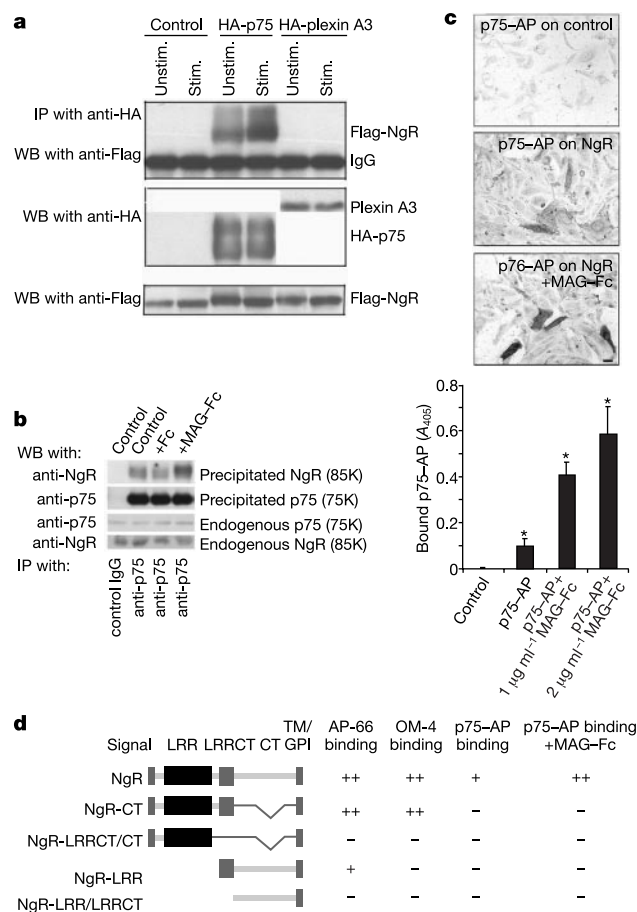


Figure 1 p75 and NgR form receptor complexes. **a**, Co-immunoprecipitation of p75–NgR from Flag-tagged, NgR-expressing cells transfected with vector, HA-p75 or HA-plexin A3, and mock-treated (Unstim.) or treated with MAG–Fc (Stim.). **b**, Co-immunoprecipitation of p75–NgR from CGNs treated with or without Fc or MAG–Fc. **c**, Visualization and quantification of p75–AP binding to NgR-expressing cells. Asterisk, $P < 0.05$ by Student's t -test comparing bound p75–AP under various conditions with control. Scale bar, 10 μ m. **d**, Summary diagram of AP fusion proteins binding to cells expressing different truncations of NgR. Signal, signal peptide; TM/GPI, transmembrane domain/GPI anchor.

precipitated together (Fig. 1b). Treatment of both the NgR-expressing CHO cells (Fig. 1a) and CGNs (Fig. 1b) with a soluble MAG protein (MAG-Fc) containing the extracellular domain of rat MAG and the human immunoglobulin- γ (IgG) Fc fragment^{3,4,8} enhanced the formation of NgR-p75 complexes, suggesting that MAG is able to induce the formation of this complex.

To confirm these initial observations, we used a cell-surface binding assay to monitor directly the NgR-p75 interaction. As shown in Fig. 1c, an alkaline phosphatase (AP) fusion protein containing the extracellular domain of p75 (p75-AP), but not AP protein alone (data not shown), was able to bind specifically to CHO cells expressing NgR, but not to control CHO cells. Consistent with the co-immunoprecipitation results in Fig. 1a and b, the binding of p75-AP to NgR-expressing cells was enhanced by the addition of MAG-Fc in a dose-dependent manner (Fig. 1c). COS cells expressing full-length p75 did not bind MAG-Fc and other known NgR ligands (data not shown). Moreover, although p75 has been shown previously to affect the binding of neurotrophins to Trk receptors⁹, we found that p75 overexpression did not enhance the binding of MAG, Nogo-66 or OMgp to NgR-expressing cells (data not shown). Thus, it is likely that p75 and NgR form a receptor complex and that ligand treatment enhances the NgR-p75 interaction.

We next determined the structural basis of the interaction between p75 and NgR. The p75-AP fusion protein lacking the transmembrane and intracellular domains of p75 was able to bind to full-length NgR (Fig. 1c), suggesting that the interaction of p75 with NgR is mediated through its extracellular domain. The amino-terminal region of NgR covering eight leucine-rich repeats (LRR) and the LRR carboxy-terminal domain (LRRCT) is sufficient to interact with MAG, OMgp and Nogo-66 (refs 1–4). However, we found that both the N-terminal LRR and LRRCT, and the C-terminal (CT) domains were indispensable for interacting with

p75 (Fig. 1d). This provides a structural explanation for our previous observations⁴ that a truncated NgR lacking the unique C-terminal region neutralized the inhibitory activity of MAG, presumably because it can bind ligands but not p75.

To examine whether all of the known NgR ligands require p75, we used a neurite outgrowth assay to evaluate the outgrowth responses to individual inhibitors in neurons from mice carrying a mutation in the *p75* gene¹⁰. Consistent with a previous report⁷, neurite outgrowth of dorsal root ganglion (DRG) neurons from *p75*^{-/-} mice was not inhibited by MAG-Fc (data not shown). Similarly, although both a glutathione *S*-transferase (GST) fusion protein containing the extracellular domain of Nogo-A (GST-66) and an AP-OMgp fusion protein (OM-4) efficiently inhibited neurite outgrowth from wild-type neurons, neither of these proteins restricted neurite outgrowth from *p75*^{-/-} mice (Fig. 2), suggesting that p75 is required for the inhibitory activities of these NgR ligands. Furthermore, we found that *p75*^{-/-} neurons were insensitive to the inhibition of central nervous system (CNS) myelin (Fig. 2), supporting the idea that the three NgR-p75 ligands account for most of the inhibitory activities associated with CNS myelin². In our assays no neurotrophins were added to the cultures. Moreover, addition of nerve growth factor (NGF; 100 ng ml⁻¹) or brain-derived neurotrophic factor (BDNF; 50 ng ml⁻¹) did not significantly affect the inhibition of neurite outgrowth by myelin or individual NgR ligands (data not shown). Thus, the neurite outgrowth responses we observed are probably independent of neurotrophin-related effects.

If the NgR-p75 interaction is important in mediating the inhibitory activities of the myelin inhibitors, reagents that interfere with such an interaction should affect the neurite outgrowth responses to these inhibitors. We first tested the ability of p75-Fc, a Fc fusion protein harbouring the extracellular domain (amino acids 1–250) of human p75, to serve as a competitor for NgR

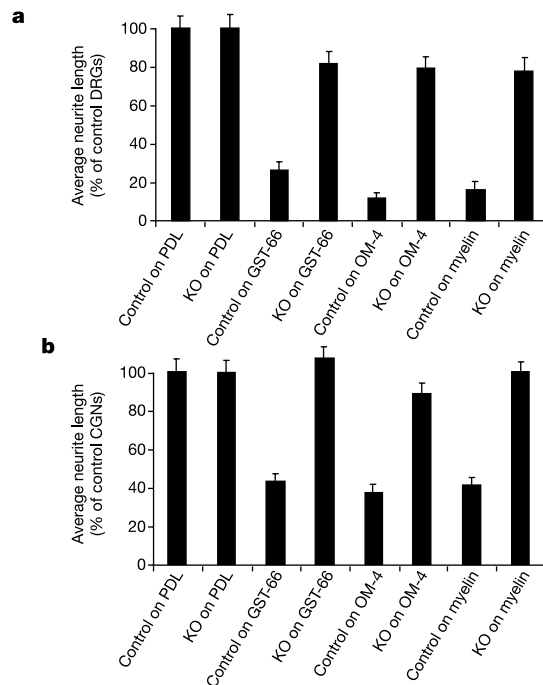


Figure 2 p75 is required for the inhibitory activities of myelin inhibitors. **a, b**, Quantification of neurite length from adult DRG neurons (**a**) or P7 CGNs (**b**) from wild-type (control) and *p75*^{-/-} (knockout, KO) mice on different immobilized substrates. The mean $\pm$ s.e.m. from a total of 1,211 (CGNs) or 923 (DRG) neurons from 3–4 experiments are presented. Statistical analysis was by one-way analysis of variance (ANOVA)

($F = 37.55$, degrees of freedom, d.f. = 1,210, $P < 0.0001$ for CGNs; $F = 41.64$, d.f. = 922, $P < 0.0001$ for DRGs), using a post-hoc Fisher protected test. All of the knockout groups on inhibitory substrates are significantly different from controls plated on the same substrates.

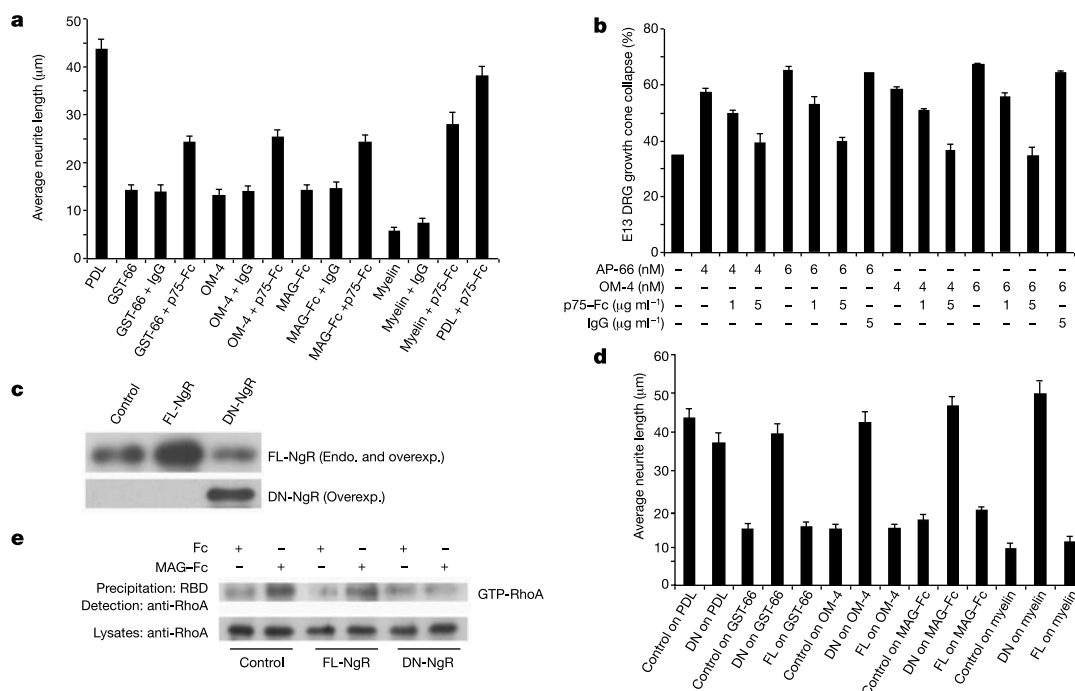


Figure 3 The p75–NgR interaction is required for the inhibitory responses to myelin inhibitors. **a**, Neurite lengths (mean $\pm$ s.e.m. from a total of 2,907 neurons from 3–4 experiments) of CGNs on immobilized substrates. Statistical analysis was by one-way ANOVA ($F = 42.18$, d.f. = 2,906, $P < 0.0001$), using a post-hoc Fisher protected test. All groups except for PDL + p75–Fc are significantly different from the PDL control. For groups cultured on inhibitory substrates, only those with the addition of p75–Fc are significantly different from groups cultured alone or with the addition of control IgG proteins. **b**, Growth cone collapse induced by AP-66 or OM-4. Statistical analysis was by one-way ANOVA ($F = 47.61$, d.f. = 29, $P < 0.0001$), using a post-hoc Fisher protected test. All groups except those with a higher concentration of p75–Fc are significantly

different from untreated controls. **c**, Expression of endogenous (Endo.) or overexpressed (Overexp.) NgR in control CGNs and those infected with retroviruses expressing full-length (FL) or truncated (dominant negative, DN) NgR. **d**, Neurite lengths (mean $\pm$ s.e.m. from a total of 2,184 neurons from 3–4 experiments) quantified from control or retrovirus-infected neurons. Statistical analysis was by one-way ANOVA ($F = 57.25$, d.f. = 2,183, $P < 0.0001$) using a post-hoc Fisher protected test. All groups except cells infected with DN–NgR and on inhibitory substrates are significantly different from the PDL controls. **e**, Affinity precipitation of GTP–RhoA from lysates of control or retrovirus-infected CGNs treated with or without MAG–Fc.

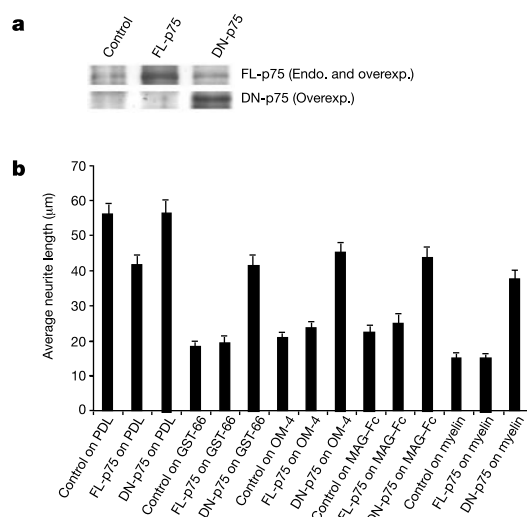


Figure 4 The intracellular domain of p75 is required for p75–NgR signalling. **a**, Expression of endogenous (Endo.) or overexpressed (Overexp.) p75 in control CGNs and those transduced with lentiviruses expressing full-length (FL) or truncated (DN) p75. **b**, Neurite lengths (mean $\pm$ s.e.m. from a total of 2,136 neurons from 3–4 experiments) quantified from control or lentivirus-transduced CGNs on immobilized substrates. Statistical analysis was by one-way ANOVA ($F = 35.75$, d.f. = 2,135, $P < 0.0001$) using a post-hoc Fisher protected test. All groups except cells transduced with DN–p75 and cultured on inhibitory substrates are significantly different from the PDL controls.

binding, and found that p75–Fc blocked the inhibitory activities of recombinant Nogo-66, OMgp and MAG, as well as CNS myelin (Fig. 3a). In an independent assay, p75–Fc also efficiently abolished the activities of soluble Nogo-66 and OMgp proteins in inducing growth cone collapse of embryonic day 13 (E13) chick DRG neurons in a dose-dependent manner (Fig. 3b). Similarly, we found that p75–Fc attenuated the activity of soluble MAG–Fc in inhibiting neurite outgrowth from dissociated E13 DRG neurons (data not shown). To provide complementary evidence for the role of the NgR–p75 interaction in mediating these inhibitory signals, we took advantage of the fact that early postnatal CGNs are susceptible to infection by recombinant retroviruses¹¹, and infected these neurons with retroviruses expressing a C-terminal-domain-truncated version of NgR, which retains the ability to bind all of the known inhibitory ligands but not p75 (ref. 4 and Fig. 1c). On overexpression in rat CGNs (Fig. 3d), this truncated NgR, but not full-length NgR, blocked the responses elicited by immobilized myelin and individual inhibitors (Fig. 3d). Together, these results indicate that the extracellular domain of p75 mediates the transduction of the inhibitory signals through its interaction with NgR.

If p75 functions as a transmembrane signal transducer for the NgR–p75 complex, its cytoplasmic domain should be required for its activity. To test this possibility, we used recombinant lentivirus-mediated gene transfer to express full-length p75 or a truncated p75 lacking the intracellular domain in postnatal day (P)7–9 rat CGNs (Fig. 4a). As shown in Fig. 4b, whereas full-length p75 did not

significantly change the responsiveness of transduced neurons to myelin and to the individual inhibitors, truncated p75 allowed robust neurite outgrowth on each of these inhibitory substrates, suggesting that the intracellular domain of p75 is required to mediate these myelin-associated inhibitory activities. Furthermore, MAG is known to activate the small GTPase RhoA in a p75-dependent manner to exert its inhibitory activity^{7,12}. Along the same lines, we found that introduction of dominant negative, but not wild-type, NgR into CGNs blocked the MAG-Fc-induced activation of RhoA (Fig. 3e). Taken together, these results suggest that NgR-p75 receptor complexes transduce the inhibitory signals into the interior of responding neurons.

Although it remains to be determined whether other proteins are present in the receptor complex that mediates the inhibitory activities of myelin components, our data suggest that p75 is a required component that participates in propagating the inhibitory signals into responding neurons. Consistent with this hypothesis, enhanced axon growth has been observed in the myelinated portions of the CNS in p75 mutant mice¹³. Although p75 expression declines to background levels in the adult CNS, an upregulation of p75 expression in injured neurons occurs as a result of lesions (refs 14, 15, and data not shown). Thus, suppressing the injury-induced p75 upregulation and intervening with p75 signalling after CNS axonal injury may considerably alleviate myelin-dependent inhibition of axonal regeneration. □

Methods

Binding experiments

Sequence encoding the extracellular domain (amino acids 30–251) of rat p75 was subcloned into the expression vector AP-5 to express a p75-AP fusion protein tagged with both a polyhistidine and an Myc epitope in COS cells. Cell-surface binding with p75-AP and other AP proteins was performed as described previously^{16,17}. For visualization of bound proteins, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate was used as AP substrate. For quantification of bound AP fusion proteins, p-nitrophenyl phosphate was used as an AP substrate, and total retained AP activity was measured at an absorbance of 405 nm (A_{405}) as described^{16,17}, from three independent experiments.

Co-immunoprecipitation

Control CHO cells or those stably expressing Flag-tagged NgR² were transfected with control or expression constructs for HA-tagged p75 by the Lipofectamine method. After 2 days, cells were lysed with ice-cold immunoprecipitation buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 200 mM NaCl, 1% NP-40) in the presence of protease inhibitors and 2 μ g ml⁻¹ orthovanadate. Some cells were incubated with soluble MAG-Fc (2 μ g ml⁻¹) for 15 min, before lysis, immunoprecipitation, and western blotting with the indicated antibodies. To detect the NgR-p75 complexes in primary neurons, cultured P7 CGNs were pretreated with or without Fc or MAG-Fc (both at 2 μ g ml⁻¹). The lysates were immunoprecipitated with control IgG or anti-p75 antibodies and western blotted with anti-NgR antibodies (Santa Cruz Biotechnology) or anti-p75 antibodies (Chemicon).

Generation of recombinant proteins and viruses

GST-66 was expressed in and purified from *Escherichia coli* as described previously¹⁸. As a construct for polyhistidine-tagged OMgp-AP protein (amino acids 33–204, designated as OM-4) resulted in higher levels of protein expression and OM-4 is functionally comparable to OM-his², we used OM-4 in this study. MAG-Fc, p75-Fc and human IgG were purchased from R&D Systems. Myelin was prepared from the white matter of bovine brain according to established protocols¹⁹.

To make recombinant retroviruses expressing full-length or truncated NgR, the respective coding complementary DNAs were first subcloned into pBabe-puro. The resultant constructs were co-transfected into 293T cells together with expression constructs for vesicular stomatitis virus (VSV-G) and gag/pol (gifts of J. Walsh and R. Mulligan). Collected conditioned medium was used to infect P5 rat CGNs for 24 h. The CGNs were then replated onto culture surfaces coated with poly-D-lysine (PDL), myelin, or the individual inhibitory proteins, and cultured for an extra 24 h before fixation and immunostaining. Recombinant lentiviruses expressing the p75 proteins were made by the ViraPower Lentiviral Expression System (Invitrogen) following the manufacturer's protocols. Viral stocks were used to transduce P6–7 rat CGNs, and the neurons were replated 48 h later onto various substrates as described above. The overexpression achieved through both retroviruses and lentiviruses resulted in more than 95% infection efficiency.

Neurite outgrowth and growth cone collapse assays

Neurite outgrowth assays were performed as described previously^{2,20}. Briefly, P7–9 rat or mouse CGNs or adult mouse DRG neurons were dissected, dissociated, and plated at a density of 10⁶ cells per ml on immobilized substrates (PDL, GST-66 (150 ng cm⁻²), OM-4

(100 ng cm⁻²), MAG-Fc (100 ng cm⁻²) and myelin (50 ng cm⁻²)) in the absence or the presence of control IgG or p75-Fc (5 μ g ml⁻¹). Cells were cultured for 24 h before fixation with 4% paraformaldehyde and staining with a neuronal-specific anti- β -tubulin antibody (Tuj-1, Covance). Neurite lengths for both the CGNs and the DRG neurons were quantified as described previously². All neurites from individual DRGs were measured and the numerical lengths averaged to obtain a single neurite length for individual neurons. Neurite lengths were measured from at least 150 neurons per condition, from duplicate wells per experiment, and from three independent experiments and quantified as described previously²¹. As a positive control, a specific inhibitor of Rho-associated protein kinase (ROCK), Y-27632 (ref. 22), neutralizes the inhibition of neurite outgrowth by myelin and the individual myelin components (data not shown), as it has been shown that RhoA-ROCK mediates the inhibitory activities of MAG and perhaps other myelin inhibitors^{23,24}.

Chick E13 DRG neurons were isolated and cultured as described previously^{1,2}. DRG explants cultured overnight were used for growth cone collapse assays. To assess the effects of p75-Fc treatment, cultures were pre-incubated with indicated amounts of p75-Fc for 30 min before treatment with individual test proteins for an additional 1 h. The means $\pm$ s.e.m. from over 400 growth cones per condition in three separate experiments are presented. p75 and NgR proteins were detected in both neurites and somas of cultured DRG neurons by immunocytochemistry.

RhoA assay

CGNs were infected with retrovirus expressing full-length or dominant negative NgR, or left uninfected; 42–48 h later these cells were stimulated in the presence of Fc or MAG-Fc for 15 min. GTP-RhoA was precipitated by using beads with GST-Rho-binding domain (RBD) of rhotekin, and detected with anti-RhoA antibodies following manufacturer's guidelines (ref. 25; Upstate Biotechnology).

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The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease

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Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, which are approved for cholesterol reduction, may also be beneficial in the treatment of inflammatory diseases^{1–3}. Atorvastatin (Lipitor) was tested in chronic and relapsing experimental autoimmune encephalomyelitis, a CD4⁺

Th1-mediated central nervous system (CNS) demyelinating disease model of multiple sclerosis^{4,5}. Here we show that oral atorvastatin prevented or reversed chronic and relapsing paralysis. Atorvastatin induced STAT6 phosphorylation and secretion of Th2 cytokines (interleukin (IL)-4, IL-5 and IL-10) and transforming growth factor (TGF)- β . Conversely, STAT4 phosphorylation was inhibited and secretion of Th1 cytokines (IL-2, IL-12, interferon (IFN)- γ and tumour necrosis factor (TNF)- α) was suppressed. Atorvastatin promoted differentiation of Th0 cells into Th2 cells. In adoptive transfer, these Th2 cells protected recipient mice from EAE induction. Atorvastatin reduced CNS infiltration and major histocompatibility complex (MHC) class II expression. Treatment of microglia inhibited IFN- γ -inducible transcription at multiple MHC class II transactivator (CIITA) promoters and suppressed class II upregulation. Atorvastatin suppressed IFN- γ -inducible expression of CD40, CD80 and CD86 co-stimulatory molecules. L-Mevalonate, the product of HMG-CoA reductase, reversed atorvastatin's effects on antigen-presenting cells (APC) and T cells. Atorvastatin treatment of either APC or T cells suppressed antigen-specific T-cell activation. Thus, atorvastatin has pleiotropic immunomodulatory effects involving both APC and T-cell compartments. Statins may be beneficial for multiple sclerosis and other Th1-mediated autoimmune diseases.

In 1995, it was reported that pravastatin treatment of cardiac transplant recipients was associated with a reduction in haemodynamically significant rejection episodes and increased survival, independent of its cholesterol-lowering effects¹. Subsequent studies demonstrated that certain statins could inhibit production of specific pro-inflammatory molecules^{2,3,6}. Lovastatin inhibited production of TNF- α and inducible nitric oxide synthetase (iNOS) by microglia and astrocytes². MHC class II expression is central to immune regulation in T-cell-mediated autoimmune disease^{5,7,8}. Statins prevented IFN- γ -inducible MHC class II expression on non-professional APC⁹, suggesting that statins might inhibit antigen presentation to pro-inflammatory Th1 cells. In that study, it was observed that statins inhibited IFN- γ -inducible expression of the MHC class II transactivator (CIITA), the master regulator for MHC class II expression¹⁰. In endothelial cells, IFN- γ -inducible CIITA transcription was inhibited at CIITA promoter (p) IV¹¹, which

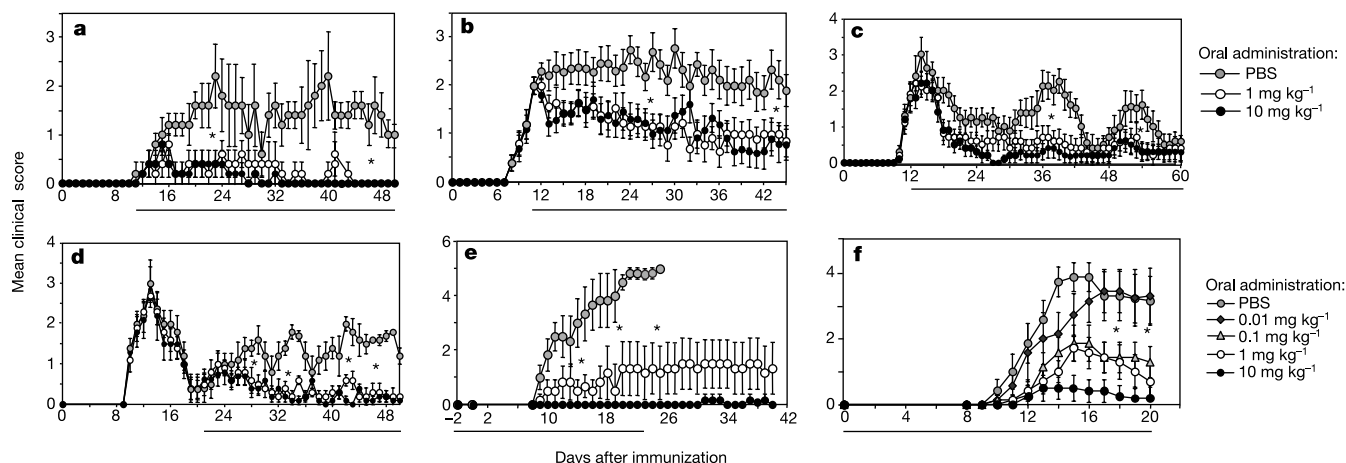


Figure 1 Atorvastatin treatment inhibits or reverses chronic and relapsing EAE. **a**, Oral atorvastatin ameliorated MOG p35–55-induced EAE in C57BL/6 mice when administered at EAE onset (within one day of initial symptoms), or **b**, after acute EAE was established. **c**, Oral atorvastatin prevented exacerbations of relapsing EAE induced by immunization of SJL/J mice with PLP p139–151. **d**, Relapsing EAE in SJL/J mice was reversed when atorvastatin treatment began at the onset of the first relapse. **e**, Limited EAE development in MBP Ac1–11-specific TCR transgenic mice after atorvastatin treatment was

discontinued. **f**, PLP p139–151-induced EAE in SJL/J mice was significantly reduced by 0.1 mg kg^{−1}, but not 0.01 mg kg^{−1} atorvastatin. Number of mice per group in each experiment: **a** (7), **b** (14), **c** (10), **d** (10), **e** (7) and **f** (7). In **a–d** mice were scored and randomized immediately before first treatment. Open circle, 1 mg kg^{−1} atorvastatin; filled circle, 10 mg kg^{−1} atorvastatin; shaded circle, vehicle only (PBS). Solid bars beneath each panel indicate atorvastatin treatment. **P* value < 0.001, comparison of either atorvastatin-treated group with vehicle-only (PBS)-treated group.

suggested to those authors that statins selectively inhibited CIITA transcription at pIV. Statins inhibited lymphocyte secretion of matrix metalloproteinase-9 (MMP-9) (ref. 3), an enzyme involved in basement membrane degradation¹². These observations suggest that statins may be beneficial in multiple sclerosis (MS). Currently approved MS treatments, which are administered parenterally, are only partly effective¹³ and are often limited by side-effects or toxicities. Statins are administered orally, are well tolerated and, in general, are considered safe.

Here we examine the immunomodulatory effects of atorvastatin in chronic and relapsing experimental autoimmune encephalomyelitis (EAE). We chose atorvastatin, the most widely prescribed statin¹⁴, with one of the most favourable safety profiles of any statin available¹⁵, because it inhibited CIITA transcription and class II expression more potently than other statins tested *in vitro*⁹. In humans 80 mg d⁻¹ of atorvastatin is the highest recommended dose for treatment of hypercholesterolaemia¹⁵. For an individual who weighs 70 kg this dose equals 1.1 mg kg⁻¹. Pharmacokinetic data in rodents indicate that larger atorvastatin doses are required to

achieve similarly effective concentrations^{16,17}. Therefore, for most experiments, we used two atorvastatin doses: 1 mg kg⁻¹ and 10 mg kg⁻¹. When atorvastatin treatment was started at clinical onset in MOG p35–55-induced EAE in C57BL/6 mice, clinical symptoms were significantly reduced in both treatment groups (Fig. 1a). When treatment was started during the acute phase, chronic EAE was ameliorated (Fig. 1b). In contrast with MOG p35–55, which causes chronic EAE, PLP p139–151 induces relapsing–remitting EAE in SJL/J mice. Atorvastatin administration prevented relapses when treatment began during the acute EAE phase (Fig. 1c) and reversed relapsing paralysis when administration was initiated at the onset of the first relapse (Fig. 1d). Atorvastatin also suppressed EAE induction in MBP Ac1–11-specific T-cell antigen receptor (TCR) transgenic mice (Fig. 1e), which develop fulminant EAE¹⁸. After atorvastatin was discontinued these did not develop fulminant EAE, whereas mice in the vehicle-only (PBS)-treated group succumbed to severe EAE. Thus, atorvastatin was effective in treatment at separate stages in three different murine EAE models. Although 1 mg kg⁻¹ and 10 mg kg⁻¹ doses were effective, we also

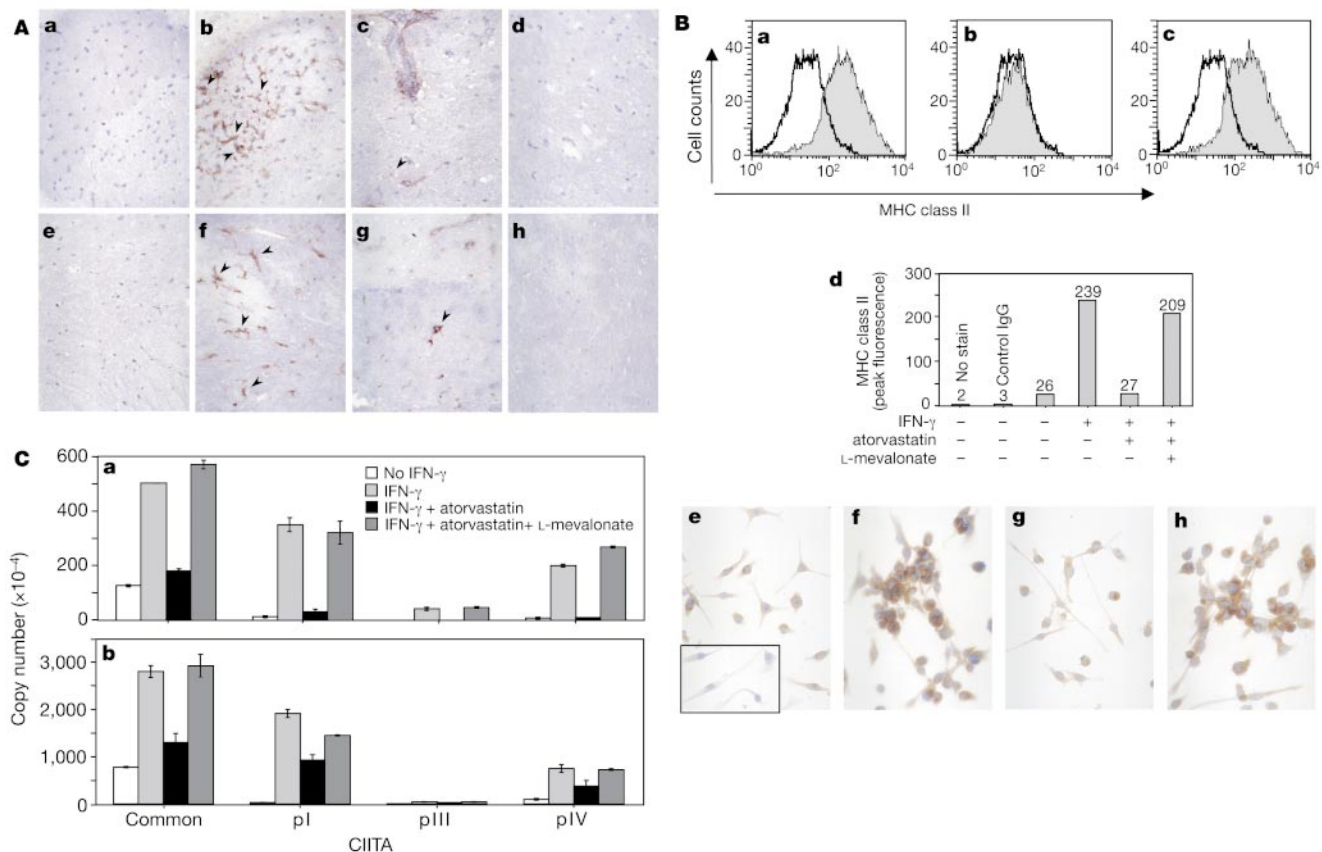


Figure 2 Atorvastatin treatment suppresses *in vivo* and *in vitro* MHC class II expression on microglia. **A**, MHC class II expression was reduced in the CNS white matter of SJL/J mice (**a–d**) and C57BL/6 mice (**e–h**) treated for EAE with atorvastatin. MHC class II molecules were rarely detected in naive CNS (**a** and **e**). High-level class II expression was detected on cells (microglia) within, or adjacent to, EAE lesions in PBS-treated mice (**b** and **f**). MHC class II expression was downregulated in the CNS of mice treated with either 1 mg kg⁻¹ atorvastatin (**c** and **g**) or 10 mg kg⁻¹ atorvastatin (**d** and **h**). Original magnification $\times 160$. Arrowheads indicate stained cells. **B**, Atorvastatin inhibits IFN- γ -inducible MHC class II expression on microglia. Flow cytometric analysis of cell-surface class II molecules on untreated or IFN- γ -treated (100 units ml⁻¹, 48 h) EOC 20 microglia (**a**). Atorvastatin (10 μ M) inhibited IFN- γ -inducible class II expression (**b**), whereas L-mevalonate (100 μ M) reversed inhibition (**c**). **d**, The mean peak fluorescence intensity for MHC class II

expression shown in **a–c** and for unstained and isotype-matched control IgG stained samples. Immunohistochemical staining for MHC class II molecules on untreated (**e**) or IFN- γ -treated (**f**) microglia. Suppression of IFN- γ -inducible class II expression on cultured microglia by atorvastatin (**g**) was reversed by addition of L-mevalonate (**h**). Inset shows isotype-matched control IgG staining. Original magnification $\times 160$. **C**, Atorvastatin inhibits multiple CIITA promoters from directing IFN- γ -inducible CIITA transcription in EOC microglia (**a**) and primary B10.PL microglia (**b**). Atorvastatin inhibited overall (common) IFN- γ -inducible CIITA transcription. IFN- γ -inducible CIITA transcription directed by pI, pIII and pIV was inhibited by atorvastatin. L-Mevalonate reversed inhibition of overall CIITA transcription and transcription initiated by each promoter. Common CIITA and promoter-specific transcripts were measured by quantitative real-time RT-PCR.

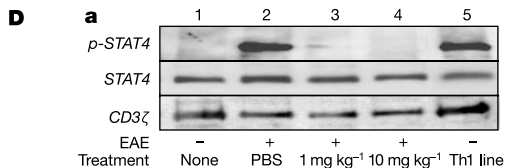


Figure 3 Atorvastatin inhibits T-cell proliferation and promotes a Th2 response. **A**, *In vivo* atorvastatin treatment of MOG p35–55-immunized C57BL/6 mice (**a**), PLP p139–151-immunized SJL/J mice (**b**) and MBP Ac1–11-immunized MBP Ac1–11-specific Tg mice (**c**) suppressed recall splenic proliferative responses. Splenocytes were isolated from mice treated *in vivo* for 12 days: vehicle-only (PBS), 1 mg kg⁻¹ atorvastatin or 10 mg kg⁻¹. **B**, *In vivo* atorvastatin treatment suppressed secretion of IL-2 (**a–c**), IL-12 (**d–f**), IFN- γ (**g–i**) and TNF- α (**j–l**), whereas secretion of IL-4 (**m–o**), IL-5 (**p–r**), IL-10 (**s–u**) and TGF- β (**v–x**) was increased. **C**, Atorvastatin treatment of naive CNS autoantigen-specific Th0 cells promoted Th2 differentiation. Atorvastatin treatment (10 μ M) of unprimed Th0 cells from MBP Ac1–11-specific B10.PL Tg mice suppressed proliferation (**a**) and secretion of IL-2 (**b**), IL-12 (**c**), IFN- γ (**d**) and TNF- α (**e**), whereas secretion of IL-4 (**f**), IL-5 (**g**), IL-10 (**h**) and

TGF- β (**1**) was augmented. Addition of L-mevalonate (100 μ M) prevented atorvastatin-induced suppression of proliferation (**a**) and induction of the Th2 'bias' (**b–i**). **D**, *In vivo* atorvastatin treatment suppressed STAT4 phosphorylation (**a**) and promoted STAT6 phosphorylation (**b**) by T cells. Protein extracts were isolated from lymph nodes of naive SJL/J mice (lane1), draining lymph nodes from PLP p139–151-immunized mice with EAE (PBS treated, lane 2), PLP p139–151-immunized mice treated with 1 mg kg⁻¹ atorvastatin (lane 3) or with 10 mg kg⁻¹ atorvastatin (lane 4). An SJL/J PLP p139–151 specific Th1 line served as a positive control for STAT4 phosphorylation (**a**, lane 5). Naive SJL/J lymph node cells treated with recombinant IL-4 *in vitro* served as a positive control for STAT6 phosphorylation (**b**, lane 5). Phosphorylated (p) STAT4 and STAT6 proteins were detected by Western blot analysis. Anti-CD3 ζ served as a protein-loading control.

tested two lower doses: 0.1 mg kg^{-1} and 0.01 mg kg^{-1} in PLP p139–151-induced EAE in SJL/J mice (Fig. 1f). The dose of 0.1 mg kg^{-1} significantly suppressed clinical EAE, whereas 0.01 mg kg^{-1} was not significantly effective through most of the observation period.

Atorvastatin-treated and vehicle-only-treated mice were examined for histological EAE. There was a large reduction in the number of inflammatory lesions in brains and spinal cords from mice treated with either 1 mg kg^{-1} or 10 mg kg^{-1} atorvastatin (Table 1 and Supplementary Information), although infiltration was reduced to a greater extent in mice treated with 10 mg kg^{-1} . Thus, clinical EAE suppression correlated with a reduction in histological EAE. A significant reduction in CNS class II expression was observed in atorvastatin-treated mice (Fig. 2A). Class II expression on microglia was also reduced in atorvastatin-treated mice (Fig. 2A) and to a greater extent in mice treated with 10 mg kg^{-1} than in those treated with 1 mg kg^{-1} (Fig. 2A, c, d, g and h), suggesting that *in vivo* class II reduction on microglia occurred in a dose-dependent fashion.

CIITA is differentially regulated by non-homologous promoters¹¹. A study reported that statins suppressed IFN- γ -inducible CIITA transcription by selective inhibition at CIITA pIV⁹. Although those authors observed that statins inhibited IFN- γ -inducible class II expression on different non-professional APC, they examined promoter-specific CIITA transcription using only endothelial cells, non-professional APC that use primarily pIV, and they only examined pIV. However, other CIITA promoters can be used to direct IFN- γ -inducible CIITA transcription^{19–21}. IFN- γ -inducible CIITA transcription in bone-marrow-derived (haematopoietic) APC, including microglia and monocytes, is directed predominantly by pI²⁰. As shown in Fig. 2B, atorvastatin inhibited IFN- γ -inducible class II expression on microglia, and this effect was reversed by L-mevalonate. CIITA pI and, to a lesser extent, pIV accounted for most of the IFN- γ -inducible CIITA transcripts in

untreated microglia (Fig. 2C). Atorvastatin prevented nearly all IFN- γ -inducible CIITA transcription directed by each CIITA promoter and this inhibition was reversed by exposure to L-mevalonate. These results clearly indicate that statins are not selective for pIV, but inhibit IFN- γ -inducible CIITA transcription in general.

As EAE is mediated by Th1 cells^{4,5}, we examined whether T-cell activation and Th regulation was altered in atorvastatin-treated mice. Compared with PBS-treated mice, *in vivo* treatment with atorvastatin suppressed the recall responses to the encephalitogenic myelin peptide used in all three models (Fig. 3A, a–c). Inhibition of proliferation appeared dose related. Culture supernatants were examined for Th1 cytokines (IL-2, IL-12, IFN- γ and TNF- α) and Th2 cytokines (IL-4, IL-5 and IL-10). As shown in Fig. 3B, atorvastatin treatment was associated with a reduction in secretion of IL-2 (panels a–c), IL-12 (d–f), IFN- γ (g–i) and TNF- α (j–l). In contrast, secretion of IL-4 (panels m–o), IL-5 (p–r), IL-10 (s–u) and transforming growth factor TGF- β (v–x), an immunoregulatory cytokine sometimes secreted concomitantly with Th2 cytokines, was increased. Thus, atorvastatin induced a Th2 bias in all three murine EAE models.

We addressed whether atorvastatin promoted Th2 differentiation of naive Th0 cells. Splenocytes from unprimed MBP Ac1–11 specific TCR transgenic mice, which contain a population of Th0 cells, were activated by MBP Ac1–11 in the presence or absence of atorvastatin. There were no significant differences in viability between treated and untreated splenocytes. As shown in Fig. 3C, atorvastatin treatment of naive Th0 cells suppressed proliferation (panel a) and secretion of Th1 cytokines (b–e), whereas secretion of Th2 cytokines (f–h) and TGF- β (i) was enhanced. Thus, atorvastatin treatment promoted Th2 differentiation. Inhibition of proliferation and Th1 cytokine secretion, as well as induction of Th2 cytokine secretion by atorvastatin, was reversed by L-mevalonate (Fig. 3C, a–i), the product of HMG-CoA reductase, indicating that these effects

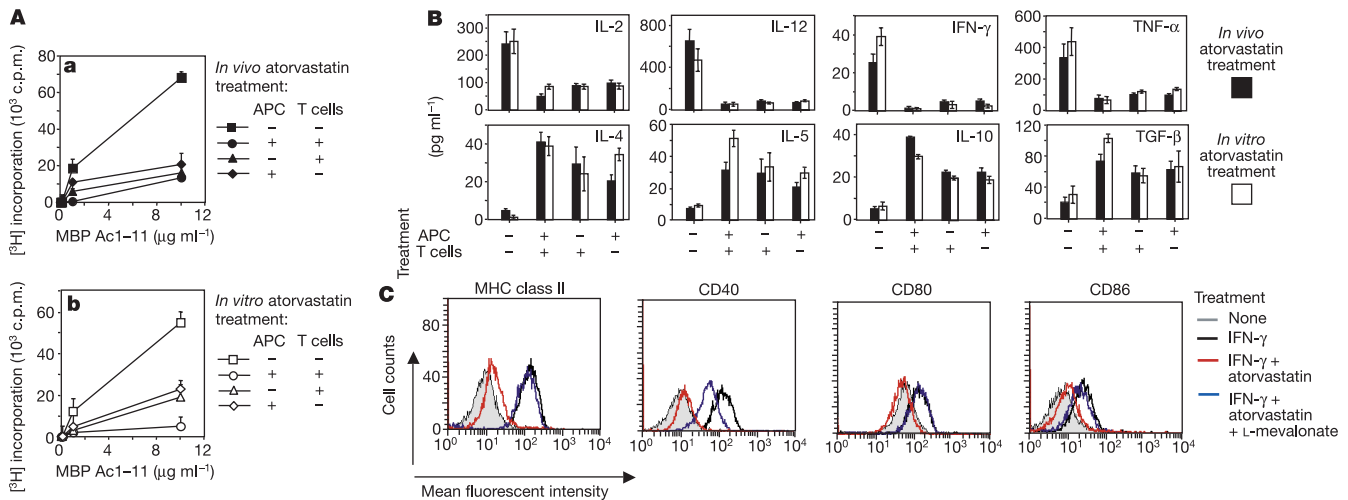


Figure 4 Atorvastatin has immunomodulatory effects on both APC and T cells. **A**, MBP Ac1–11-specific T-cell proliferation was examined using untreated or atorvastatin-treated APC with either untreated or atorvastatin-treated Th0 cells. **(a)** APC were isolated from B10.PL mice treated with either PBS or 10 mg kg^{-1} atorvastatin daily for 10 days. CD3 $^{+}$ T cells were purified from MBP Ac1–11-specific TCR transgenic mice that were treated with either PBS or 10 mg kg^{-1} atorvastatin daily for 10 days. In a reciprocal manner, atorvastatin-treated or untreated APC (5×10^5 per well) were cultured with atorvastatin-treated or untreated CD3 $^{+}$ purified Th0 cells (10^4 per well) and stimulated with MBP Ac1–11. Proliferative responses were reduced using APC or T cells isolated from atorvastatin-treated mice. **(b)** *In vitro* atorvastatin treatment of either APC or T cells alone also suppressed proliferation. Splenic APC ($6 \times 10^6 \text{ ml}^{-1}$) from B10.PL mice were cultured in the presence of $10 \mu\text{M}$ atorvastatin or media alone for 4 h at 37°C . Separately, purified CD3 $^{+}$ T cells ($6 \times 10^6 \text{ ml}^{-1}$) from MBP Ac1–11-specific TCR transgenic mice were

cultured in the presence of atorvastatin ($10 \mu\text{M}$) or media for 4 h. APC and T cells were then washed three times by centrifugation. APC were irradiated and both APC and T cells were counted. No loss in viability was detected in either atorvastatin-treated APC or T cells compared with untreated cells. In a reciprocal manner, untreated or atorvastatin-treated APC were cultured with untreated or atorvastatin-treated purified CD3 $^{+}$ and stimulated with MBP Ac1–11. Proliferative responses were reduced when either APC or CD3 $^{+}$ T cells were treated *in vitro* with atorvastatin. **B**, Inhibition of proliferation by either *in vivo* or *in vitro* treatment of APC or T cells in **A** corresponded to a Th2 cytokine pattern of secretion. **(B)**, **C**, Atorvastatin treatment of primary macrophages inhibited IFN- γ -inducible expression of class II molecules and CD40, CD80 and CD86 co-stimulatory molecules as measured by flow cytometric analysis: untreated macrophages (grey area), IFN- γ -treated ($100 \text{ units ml}^{-1}$, 48 h, black line), IFN- γ and atorvastatin ($10 \mu\text{M}$, red line). L-Mevalonate ($100 \mu\text{M}$, blue line) reversed inhibition by atorvastatin.

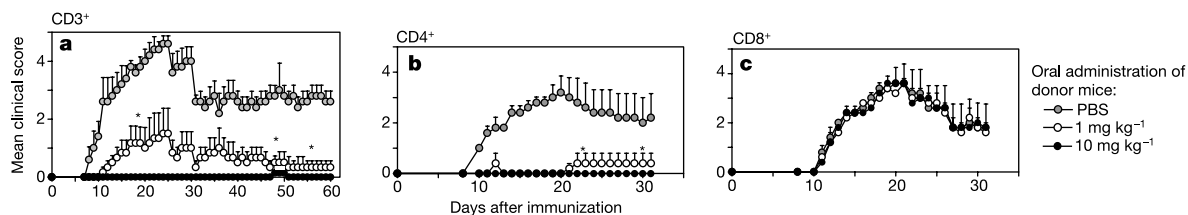


Figure 5 Adoptive transfer of atorvastatin-treated T cells prevents induction of EAE in recipient mice. Purified CD3⁺ T cells (**a**) and CD4⁺ T cells (**b**), but not CD8⁺ T cells (**c**) from atorvastatin-treated donor mice protected recipient mice from EAE induction. Donor MBP Ac 1–11-specific TCR Tg B10.PL mice were treated with either PBS, 1 mg kg⁻¹ atorvastatin or 10 mg kg⁻¹ atorvastatin for 10 days. Splenocytes were isolated and cultured in the presence of MBP Ac1–11 for 48 h. CD3⁺ T cells were isolated and injected

intravenously into recipient B10.PL mice (10⁷ T cells per mouse) (**a**). One day later, recipient mice were immunized for EAE induction. Recipient mice of PBS-treated, 1 mg kg⁻¹ atorvastatin and 10 mg kg⁻¹ atorvastatin were examined and scored for clinical EAE daily. In a separate experiment, CD4⁺ (**b**) and CD8⁺ (**c**) T cells were purified and tested in a similar manner. **P* < 0.001 when comparing atorvastatin-treated groups with PBS control groups.

were mediated through inhibition of the mevalonate pathway.

Activated (tyrosine-phosphorylated) signal transducer and activator of transcription (STAT) 4 has a key role in IL-12-dependent Th1 lineage commitment²². Conversely, STAT6 is required for IL-4-dependent Th2-dependent lineage commitment²². Thus, we examined whether atorvastatin treatment suppressed formation of activated STAT4 or induced activation of STAT6 in T cells from atorvastatin-treated or PBS-treated mice (Fig. 3D). *In vivo* atorvastatin treatment was associated with inhibition of STAT4 phosphorylation (Fig. 3D, a, lanes 3 and 4) and induction of STAT6 phosphorylation (b, lanes 3 and 4).

It was possible that the atorvastatin-induced Th2 bias reflected an alteration in APC function only. Alternatively, atorvastatin may have had direct immunomodulatory effects on both APC and T cells. To distinguish between these possibilities, we examined MBP-specific T-cell activation when only APC or T cells were exposed to atorvastatin. APC were isolated from atorvastatin-treated or PBS-treated mice. T cells that were positive for the CD3 cell-surface protein (referred to hereafter as CD3⁺ cells) were purified from atorvastatin-treated or PBS-treated mice. In a reciprocal manner, APC from atorvastatin-treated or untreated mice were cultured with CD3⁺ purified Th0 cells from atorvastatin-treated or untreated mice. Proliferation was inhibited by using either *in vivo*-treated APC or T cells (Fig. 4A, a). A Th2 cytokine pattern was also observed when using either *in vivo*-treated APC or T cells (Fig. 4B). We similarly analysed this by treating either APC or purified transgenic CD3⁺ T cells separately *in vitro*. Again, proliferation was suppressed (Fig. 4A, b) and a Th2 bias was induced (Fig. 4B) by using either atorvastatin-treated APC or CD3⁺ T cells. These results confirmed that atorvastatin exerts immunomodulatory

effects on both APC and T cells. As atorvastatin treatment suppressed class II (signal 1) upregulation on APC (Fig. 2B) and inhibited secretion of IL-12 (Fig. 3), we examined whether atorvastatin altered the APC expression of co-stimulatory (signal 2) molecules. Atorvastatin inhibited IFN-γ-inducible expression of CD40, CD80 (B7-1) and CD86 (B7-2) co-stimulatory molecules, as well as class II molecules on primary macrophages; these effects were reversed by L-mevalonate (Fig. 4C). Atorvastatin-treated macrophages suppressed proliferation of untreated, purified CD3⁺-syngeneic Th0 cells and promoted a Th2 bias (data not shown).

We examined directly whether atorvastatin-induced Th2 cells could serve as regulatory cells *in vivo*. By adoptive transfer we tested whether atorvastatin-induced Th2 cells could protect recipient mice from EAE induction. Splenocytes were isolated from B10.PL MBP Ac1–11-specific TCR Tg mice treated with either atorvastatin or PBS, then cultured with MBP Ac 1–11. Cytokine analysis confirmed a Th2 bias for lymphocytes from atorvastatin-treated mice and a Th1 bias for lymphocytes from PBS-treated mice (data not shown). CD3⁺ T cells were purified. One day after adoptive transfer, recipient B10.PL mice were immunized for EAE induction. All of the recipient mice that received lymphocytes from PBS-treated mice developed severe EAE (Fig. 5a). In contrast, recipient mice of donor lymphocytes from mice treated with 10 mg kg⁻¹ atorvastatin were almost entirely protected from EAE. Mild EAE developed in 50% of mice that received lymphocytes from donor mice treated with 1 mg kg⁻¹ atorvastatin. In a subsequent experiment, atorvastatin-treated donor T cells were separated into CD4⁺ and CD8⁺ subpopulations. The CD4⁺ T cells (Fig. 5b), but not the CD8⁺ T cells (Fig. 5c), protected recipient mice from EAE induction. These

Table 1 Atorvastatin suppresses clinical and histological EAE

Atorvastatin dose per day (mg kg ⁻¹)	Clinical EAE			Number of inflammatory foci†		
	Incidence	Mean day of onset ± s.e.m.	Mean score ± s.e.m.	Meningeal	Parenchymal	Total
a, Prevention of relapsing EAE in SJL/J mice*						
0	7/7	10 ± 1	3.3 ± 0.31	30 ± 9	25 ± 10	55 ± 20
1	3/7	13 ± 1	0.57 ± 0.3	10 ± 2	2 ± 1	12 ± 2
10	0/7	0 ± 0	0 ± 0	0.4 ± 0.44	0 ± 0	0.4 ± 0.44
b, Treatment of relapsing EAE in SJL/J mice‡						
	Day 16§	Day 36§	Day 53§	Day 16§	Day 36§	Day 53§
0	10/10	7/8	5/5	2.5 ± 0.3	2.2 ± 0.4	1.5 ± 0.25
1	9/10	4/10	2/7	2 ± 0.3	0.7 ± 0.3	0.5 ± 0.4
10	10/10	2/10	1/7	2 ± 0.27	0.3 ± 0.2	0.2 ± 0.19
				0.33 ± 0.4	0 ± 0	0.33 ± 0.4

*For EAE prevention, mice were treated daily for 16 days, starting 2 days before EAE induction.

†Brains and spinal cords from all treatment groups (**a**, prevention: five mice per group; **b**, treatment: three mice per group were examined for inflammatory foci at day 14 (**a**, prevention) or at day 36 (**b**, treatment). Results are shown as the mean number of inflammatory foci ± s.e.m.

‡Atorvastatin treatment began during acute EAE at day 12 (mean EAE score 1.78 ± 0.4) and continued for 48 days (Fig. 1c).

§Acute EAE (day 16, Fig. 1c); first relapse (day 36, Fig. 1c); second relapse (day 53, Fig. 1c).

^{||}*P* value < 0.001, comparison of either atorvastatin-treated group with vehicle-only (PBS)-treated group.

adoptive transfer studies provided evidence that atorvastatin treatment induced a population of regulatory T cells that suppressed clinical autoimmune disease.

We have shown that oral statin treatment suppressed MHC class II upregulation *in vivo*, inhibited APC upregulation of co-stimulatory molecules, and was effective in prevention or reversal of chronic and relapsing CNS autoimmune disease. We also demonstrated that statin treatment promoted differentiation of Th2 cells, which were biologically active in regulating Th1-mediated autoimmune disease. Thus, our results demonstrate that statins can have pleiotropic immunomodulatory effects involving APC and T-cell compartments.

Statins may have multiple targets in immune modulation^{2,6,9,23}. Certain statins bind LFA-1 and inhibit its interaction with ICAM-1 (ref. 23), indicating that statins can interact with a protein involved in T-cell adhesion/co-stimulation. Other recognized effects of statins are mediated through inhibition of the mevalonate pathway^{2,6}. Mevalonate is a substrate in cholesterol biosynthesis, but also participates in post-translational modification (isoprenylation) of proteins involved in cell division and maturation^{24,25}. Statins inhibited the production of proinflammatory cytokines and chemokines, effects that were reversed by mevalonate^{2,6}. We have shown that inhibition in expression of IFN- γ -inducible CIITA, class II and co-stimulatory molecules on APC, and Th2 differentiation induced by atorvastatin, are mediated through inhibition of the mevalonate pathway.

MS is a multiphasic disease²⁶. CNS inflammation and demyelination characterize the early relapsing–remitting phase, whereas neuronal loss and atrophy occur in the chronic ‘secondary progressive’ phase²⁶. Our results in this study support use in the inflammatory phase. Lovastatin, which partly suppressed acute EAE in rats when administered parenterally²⁷, inhibited production of iNOS and TNF- α ^{2,27}, pro-inflammatory molecules that are neurotoxic, suggesting that statins might be beneficial in chronic MS. Simvastatin is being tested in a small open-label trial in relapsing–remitting MS (information available at the National Multiple Sclerosis Society, New York (<http://www.nationalmssociety.org>)). MS trials using atorvastatin are being planned. Statins are attractive candidates for prophylactic treatment in patients who have experienced a single demyelinating event, a ‘clinically isolated syndrome’, and are at risk of recurrences or conversion to clinically definite MS. As statins have different mechanisms of action from currently approved MS treatments, they may be useful in combination therapy. Our results also provide a rationale for testing atorvastatin in other organ-specific Th1-mediated autoimmune diseases, including diabetes and rheumatoid arthritis. The anti-inflammatory properties described here may also contribute to the protective effects of statins in heart disease and stroke, effects traditionally attributed to cholesterol reduction. □

Methods

Animals

Female SJL/J, B10.PL and C57BL/6 mice (8–12-weeks old) were purchased from the Jackson Laboratory (Bar Harbor). MBP Ac 1–11 TCR transgenic mice, obtained from C. Janeway Jr¹⁸, were backcrossed into the B10.PL background. All animal protocols were approved by the Division of Comparative Medicine at Stanford University and the Committee of Animal Research at the University of California San Francisco, in accordance with the National Institutes of Health guidelines.

Peptides

MBP Ac1–11 (Ac-ASQKRPSQRHG), MOG p35–55 (MEVGWYRSPFSRVVHLYRNGK), PLP p139–151 (HCLGKWLGHDPKF) and the control peptide HSPV16 (DMTPADALDDRDLEM) were synthesized on a peptide synthesizer (model 9050; MilliGen) by standard 9-fluorenylmethoxycarbonyl chemistry, and purified by high-performance liquid chromatography (HPLC). Amino acid sequences were confirmed by amino acid analysis and mass spectroscopy. The purity of each peptide was greater than 95%.

Microglia and macrophages

Microglia EOC 20 cells, derived from C3H/HeJ CH-2k mice²⁸, were obtained from the

American Type Culture Collection (ATCC) and were grown as recommended using DMEM media supplemented with 1 mM sodium pyruvate, 10% (v/v) fetal calf serum (FCS) and 20% (v/v) conditioned media as a source for mouse CSF-1. These microglia stained positively for CD11b. Primary microglia were isolated from 2-day-old B10.PL mice as described previously²⁹. Primary microglia were 95% CD11b⁺ by fluorescence-activated cell sorting (FACS). Primary macrophages (peritoneal exudate cells (PEC)) were harvested from B10.PL mice 24 h after intraperitoneal injection with 1 ml of 3% (w/v) thioglycollate. PEC were cultured with media alone for 72 h, then activated with IFN- γ (100 U ml^{-1}) or treated with media alone. PEC were 98% (w/v) CD11b⁺ by FACS analysis.

Atorvastatin treatment

Atorvastatin (Pfizer Inc.) (prescription formulation) was brought into suspension in PBS. Atorvastatin was administered orally in 0.5 ml (for example, 0.04 mg ml^{-1} for 1 mg kg^{-1} dose or 0.4 mg ml^{-1} for 10 mg kg^{-1} dose) once daily using 20-mm feeding needles (Popper and Sons Inc.). PBS was administered as control. Purified atorvastatin, used for *in vitro* studies, was provided by R. Laskey (Pfizer Inc.).

Experimental autoimmune encephalomyelitis induction

EAE was induced in SJL/J mice, C57BL/6 mice or MBP Ac1–11-specific TCR transgenic mice by immunization with $100 \mu\text{g}$ of PLP p139–151, MOG p35–55 or MBP Ac1–11, respectively. All peptides were dissolved in complete Freund’s adjuvant (CFA) containing 4 mg ml^{-1} of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories) as described in ref. 5. On the day of immunization and 48 h later, C57BL/6 mice and MBP Ac1–11 TCR transgenic mice were injected with 100 ng of *Bordetella pertussis* toxin (BPT) in PBS, intravenously (i.v.). Mice were examined daily for clinical signs of EAE and scored as follows: 0, no paralysis; 1, loss of tail tone; 2, hindlimb weakness; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; 5, moribund or dead.

Adoptive transfer

B10.PL MBP Ac1–11-specific TCR Tg mice (four per group) were treated daily with either 1 mg kg^{-1} atorvastatin or 10 mg kg^{-1} atorvastatin or vehicle (PBS) only. After 12 days of oral treatment, mice were killed. Splenocytes were isolated and cultured in the presence of MBP Ac1–11 ($10 \mu\text{g ml}^{-1}$) for 48 h. Viable cells were removed by Ficoll gradient. CD3⁺, CD4⁺ or CD8⁺ T cells were purified by high-affinity negative selection (R and D Systems). Greater than 85% purity was obtained for each. One million T cells were injected intravenously into naive B10.PL recipient mice. Twenty-four hours after adoptive transfer, recipient mice were immunized with MBP Ac 1–11 for EAE induction as previously described and scored daily for clinical signs: Fig. 5a, PBS ($n = 5$), 1 mg kg^{-1} atorvastatin ($n = 6$), 10 mg kg^{-1} atorvastatin ($n = 7$); Fig. 5b and c, $n = 5$ for all groups.

Flow cytometry

Immunofluorescent staining was done as described in ref. 30. After incubation for 48 h, cells were washed with FACS buffer (PBS containing 0.1% (w/v) sodium azide and 2% (v/v) FCS) and preincubated with anti-mouse CD16/CD32 monoclonal antibody (clone 2.4G2, PharMingen) for 10 min at 4 °C to block non-specific binding to Fc receptors. Fluorochrome-conjugated monoclonal antibodies (rat anti-mouse Mac-1/CD11b-PE (M1/70, IgG2b), mouse anti-mouse MHC class II (I-A^b)-FITC (10-3.6, IgG2b), hamster anti-mouse CD40-FITC (HM40-3, IgM), hamster anti-mouse CD80-FITC (16-10A1, IgG) and rat anti-mouse CD86-FITC (GL1, IgG2a) were purchased from PharMingen. Background fluorescence was evaluated by staining the cells with isotype control antibodies: rat IgG2b-PE or FITC and mouse IgG2b-FITC (PharMingen). After incubation, cells were washed twice with FACS buffer and analysed by FACScan using CellQuest software (Becton Dickinson). Twenty thousand events were analysed.

Antigen-specific T-cell proliferation assays

Splenocytes or lymph node cells (LNC) were isolated from atorvastatin or vehicle-only-treated mice and culture *in vitro* with the specific encephalitogenic peptide (PLP p139–151, MOG p35–55 or MBP Ac1–11) used for the immunization or with either concanavalin A (con A) (positive control) or HSPV16 (negative control). Cells were cultured in 96-well microtitre plates at a concentration of $5 \times 10^6 \text{ cells ml}^{-1}$. Culture medium consisted of RPMI 1640 supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids (0.1 mM), penicillin (100 U ml^{-1}), streptomycin (0.1 mg ml^{-1}), 2-mercaptoethanol ($5 \times 10^{-3} \text{ M}$) and 1% (v/v) autologous normal mouse serum. Splenocytes and LNC from SJL/J or C57BL/6 mice were incubated for 72 h whereas cultures from MBP Ac1–11 Tg mice were incubated for 48 h. Cultures were then pulsed for 18 h with $1 \mu\text{Ci}$ per well of [³H]thymidine before harvesting. Results are shown as mean of triplicates $\pm$ s.e.m.; s.e.m.s were within 10% of the mean.

Cytokine analysis

Supernatants from splenocytes and LNC cultured in parallel with those cells used in proliferation assays tested were used for cytokine analysis. Supernatants were collected at different times for measurements of cytokine levels: 48 h for IL-2 and IL-12, 72 h for IFN- γ , TNF- α and TGF- β , and 120 h for IL-4, IL-5 and IL-10. IL-2, IL-5, IL-10, IL-12 (p70), IFN- γ and TNF- α levels were determined by using specific enzyme-linked immunosorbent assay (ELISA) kits for the corresponding cytokines according to the manufacturer’s protocols (anti-mouse OPTeia Kits, PharMingen). IL-4 was measured using a cytometric bead array kit (BD Biosciences). TGF- β was measured by ELISA (R and D Systems). Results are shown as mean of triplicates $\pm$ s.e.m.; s.e.m.s were within 10% of the mean.

Total RNA isolation

Mice were killed and perfused with 20 ml of cold sterile PBS. Total RNA was isolated from

brain tissues using Trizol reagent (Invitrogen) as recommended in the manufacturer's protocol. Microglia cell cultures were washed once with PBS, and RNA was isolated using Trizol reagent as described by the manufacturer's protocol. RNA concentration was measured by spectrophotometry at 260 nm.

Evaluation of CIITA promoter-specific mRNA expression

One-step polymerase chain reaction with reverse transcription (RT-PCR) was performed by using a master mix that was prepared with 400 mM dUTP and 200 mM each of dATP, dCTP and dGTP; 0.2 mM each oligonucleotide primer, 0.2 × SYBR green in DMSO (1% final concentration); 2.5% (v/v) glycerol; 1 U uracil *N*-glycosylase; 4 mM Mn(OAc)₂; and 5 U rTth polymerase. RT-PCR parameters: initial incubation 10 min at 45 °C with activating uracil *N*-glycosylase followed by reverse transcription 30 min at 60 °C; 50 cycles at 95 °C for 15 s and 57 °C for 30 s. β-Actin was amplified from all samples as a housekeeping gene to normalize expression. A control (no template) was included for each primer set. For quantification, a tenfold dilution series of a CIITA run-off transcript (10⁷–10² initial CIITA copies) was included in each reaction plate. Data were analysed by Sequence Detection Systems software and transferred to a Microsoft Excel spreadsheet for analysis. A calibration curve was generated by plotting CIITA (run-off transcript) for each tenfold dilution against the number of cycles required for each product to exceed a preset threshold (Ct). Ct values were compared with those obtained on a standard curve. Primers for common CIITA (nucleotides 2374–2458) were: 5'-GCCACGAGACACAGCAA and 5'-TGAGCCGGGTGCCAGGAA. The 5' (forward) promoter-specific primers were: pI CIITA (pI nt 259), 5'-CCTGACCCTGCTGGAGAA; pIII CIITA (pIII nt 112), 5'-GCATCACTCTGCTCTCTAA; pIV CIITA (pIV nt 43), 5'-TGCAGGCAGCACTCAGAA. CIITA (nt 265) reverse primers for promoter-specific transcripts were: 5'-GGGGTGGGCACTGTGA; β-actin: (301–538): 5'-CGACCTGGGGATCTTCTA and 5'-TCGTGCCCTCAGTCTTCAA.

Western blot analysis for STAT4 and STAT6 phosphorylation

Lymph nodes from control and atorvastatin-treated mice were isolated and homogenized in T-PER protein extraction buffer (Pierce Chemical Co.), with 20 mg ml⁻¹ aprotinin, 20 mg ml⁻¹ leupeptin, 1.6 mM Pefablock SC (Roche), 10 mM NaF, 1 mM Na₃VO₄ and 1 mM Na₂P₂O₇ (Sigma). Naive lymph-node cells cultured with mouse recombinant IL-4 (10 ng ml⁻¹) for 1 h served as a positive control for STAT6 activation. Protein concentrations were determined by bicinchoninic acid protein assay (Pierce). Lysates were added to 3× SDS loading buffer (Cell Signaling Technology) with 40 mM DTT. Products were separated by electrophoresis on a 4–15% SDS–PAGE gradient gel (Bio-Rad Laboratories Inc.). Pre-stained markers (Invitrogen) were used to determine molecular mass. Gels were blotted to PVDF membranes at 100 V in 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, then blocked for 1 h at room temperature with Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 and 5% (w/v) non-fat dry milk. After washing in TBS and 0.1% (v/v) Tween 20, membranes were hybridized overnight at 4 °C with anti-phospho-STAT4 (Zymed Laboratories Inc.) antibody or anti-phospho-STAT6 antibody (Cell Signaling Technology, Inc.) diluted 1:1,000 in TBS, 0.1% (v/v) Tween 20 and 5% (w/v) BSA. The membranes were then processed by ECL Plus protocol (Amersham BioSciences, Inc.) for visualization of the bands. Membranes were stripped in 100 mM 2-mercaptoethanol, 2% (w/v) SDS and 62.5 mM Tris (pH 7.4) for 30 min at 60 °C, then probed with anti-CD3ζ (PharMingen), anti-STAT4 and anti-STAT6 (BD Biosciences) as a control to verify equal protein loading. Phosphorylated (p) STAT4 and STAT6 migrated at a relative molecular mass of ~100,000.

Histopathology and immunohistochemistry

Anaesthetized mice were perfused with 20 ml cold PBS. Brains and spinal cords were fixed in 4% (w/v) paraformaldehyde and embedded in paraffin. Sections were stained with haematoxylin and eosin. Selected brain, thoracic and lumbar spinal cord sections were evaluated by an examiner blinded to the treatment status of the animal. For MHC class II evaluation, frozen sections were used. Brain tissues collected on day 14 after immunization from two to five mice in each experimental group, were embedded in Tissue-Tek O.C.T. Compound (VWR Scientific Products) quick-frozen in solid CO₂ mixed with isopentane, and maintained at –80 °C until sectioning. Cryostat sections (4–6 μm) were fixed with acetone and then labelled with mouse anti-I-A^{k,s} monoclonal antibody (mAb) 10-3.6 (PharMingen) or mouse anti-I-A^b mAb AF6-120.1 (PharMingen), using the avidin–biotin technique (Vector Laboratories). Staining was visualized by reaction with diaminobenzidine (DAB). Slides were counterstained with haematoxylin. Isotype-matched IgG and omission of the primary antibody served as negative controls. Microglia cells were grown on cover slides and treated with IFN-γ alone or with the addition of atorvastatin or atorvastatin and L-mevalonate for 48 h. Slides were then fixed with acetone and stained with anti-I-A^k (10-3.6) using the same procedure as for frozen sections.

Statistical analysis

Data are presented as mean ± s.e.m. For clinical scores, significance between each two groups was examined by using the Mann–Whitney *U* test. A value of *P* < 0.05 was considered significant. All other statistics were analysed by using a one-way multiple-range analysis of variance test (ANOVA) for multiple comparison. A value of *P* < 0.05 was considered significant.

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Recruitment and regulation of phosphatidylinositol phosphate kinase type 1 γ by the FERM domain of talin

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Membrane phosphoinositides control a variety of cellular processes through the recruitment and/or regulation of cytosolic proteins^{1–4}. One mechanism ensuring spatial specificity in phosphoinositide signalling is the targeting of enzymes that mediate their metabolism to specific subcellular sites. Phosphatidylinositol phosphate kinase type 1 γ (PtdInsPKI γ) is a phosphatidylinositol-4-phosphate 5-kinase that is expressed at high levels in brain, and is concentrated at synapses^{5,6}. Here we show that the predominant brain splice variant of PtdInsPKI γ (PtdInsPKI γ -90) binds, by means of a short carboxy-terminal peptide, to the FERM domain of talin, and is strongly activated by this interaction. Talin, a principal component of focal adhesion plaques⁷, is also present at synapses. PtdInsPKI γ -90 is expressed in non-neuronal cells, albeit at much lower levels than in neurons, and is concentrated at focal adhesion plaques, where phosphatidylinositol-4,5-bisphosphate has an important regulatory role. Overexpression of PtdInsPKI γ -90, or expression of its C-terminal domain, disrupts focal adhesion plaques, probably by local disruption of normal phosphoinositide balance. These findings define an interaction that has a regulatory role in cell adhesion and suggest new similarities between molecular interactions underlying synaptic junctions and general mechanisms of cell adhesion.

Phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) controls the nucleation of a variety of plasma-membrane-associated scaffold proteins^{4,8,9}. In presynaptic nerve terminals, a pool of PtdIns(4,5)P₂, which is regulated by the opposite actions of PtdInsPKI γ and synaptojanin, mediates clathrin coating and actin dynamics at endocytic zones^{6,10}. PtdInsPKI γ , a member of the type I PtdIns phosphate kinase family¹¹, phosphorylates PtdIns(4)-phosphate on the D-5 position of the inositol ring⁶; synaptojanin is a polyphosphoinositide phosphatase that dephosphorylates PtdIns(4,5)P₂ (refs 12, 13). Although several interactions that may account for the localization of synaptojanin within the synapse have been described^{12,14}, mechanisms underlying PtdInsPKI γ subcellular targeting are unknown.

The C-terminal region of PtdInsPKI γ undergoes alternative splicing that accounts for the inclusion or exclusion of a 28-amino-acid tail (28aa-tail), thus generating two isoforms of relative molecular mass 90,000 and 87,000 (PtdInsPKI γ -90 and PtdInsPKI γ -87), respectively⁵. As a first step towards understanding the role of the two alternatively spliced C-terminal regions of PtdInsPKI γ in intracellular localization, PtdInsPKI γ -90 and PtdInsPKI γ -87 tagged with green fluorescent protein (GFP) were expressed into NIH 3T3 cells. Both proteins were partially targeted to the plasma membrane (data not shown), but PtdInsPKI γ -90, unlike PtdInsP-

KI γ -87, was further concentrated at focal adhesion contacts, as demonstrated by its colocalization with vinculin (Fig. 1a) and focal adhesion kinase (FAK, data not shown). Furthermore, focal adhesions were disrupted in cells expressing high levels of PtdInsPKI γ -90 (see below) (Fig. 1a). When fused to GFP, the 28aa-tail of PtdInsPKI γ -90 targeted the corresponding fusion protein to focal adhesion plaques, although not as efficiently as full-length PtdInsPKI γ -90 (Fig. 1a). Consistent with the localization of transfected PtdInsPKI γ -90, endogenous PtdInsPKI γ immunoreactivity was concentrated at focal adhesion plaques in cultured astrocytes (Fig. 1b).

These findings indicate that the 28aa-tail is a focal adhesion targeting domain. To elucidate the molecular basis for this recruitment, we searched for a potential 28aa-tail binding protein. A rat brain detergent extract was affinity-purified on glutathione S-transferase (GST) fusion proteins of the C-terminal region of either PtdInsPKI γ -87 or PtdInsPKI γ -90 (Fig. 2a). A prominent band of approximately 240K was selectively retained by the C-terminal region of PtdInsPKI γ -90 (Fig. 2b) or by a GST-28aa-tail (data not shown). Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) analysis of tryptic digests from the 240K protein identified several peptides, all of which corresponded to talin 2 sequences (see Methods and Supplementary Information). This identification was confirmed by western blotting using antibodies that recognize both talin 1 and 2 (Fig. 2b and data not shown). The interaction between the 28aa-tail and talin is direct, because GST-28aa-tail, but not GST alone, bound to talin in a gel

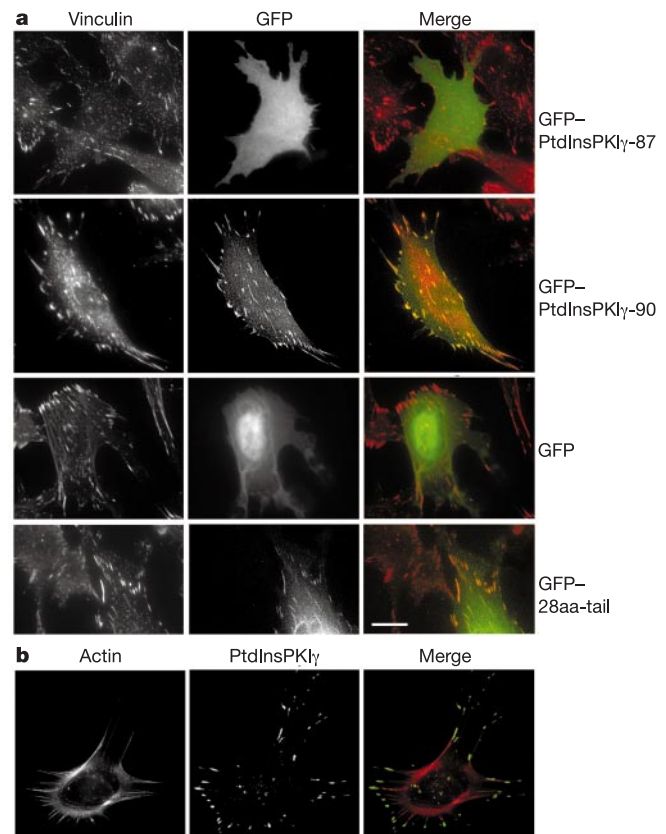


Figure 1 The 90K splice variant of PtdInsPKI γ is targeted to focal adhesion plaques in non-neuronal cells. **a**, NIH 3T3 cells were transfected with the constructs indicated (green), then fixed and counterstained for vinculin (red), a focal adhesion plaque marker. GFP-PtdInsPKI γ -90 and GFP-28aa-tail, but not GFP-PtdInsPKI γ -87 and GFP alone, are localized to focal adhesion plaques. **b**, Endogenous PtdInsPKI γ (green) is localized to sites of focal adhesion at the tip of actin stress fibres (red) in astrocytes. Scale bar, 10 μ m.

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overlay assay (Fig. 2c).

Talin consists of a 47K N-terminal globular head, which includes a FERM (4.1/ezrin/radixin/moesin) domain and a 190K C-terminal rod (Fig. 2d)^{7,15}. The binding site for the 28aa-tail of PtdInsPKI γ -90 was mapped to the head region. Thus, *in vitro* translated haemagglutinin (HA)-tagged PtdInsPKI γ -90, but not PtdInsPKI γ -87, was retained by an immobilized GST fusion protein of the head domain of talin 2 (amino acids 1–437) (Fig. 2e). The rod region of talin 2 did not include any additional binding sites for PtdInsPKI γ -90 (data not shown). GST fusion proteins of the head domains of talin 1 and 2 bound PtdInsPKI γ -90 with comparable efficiency (Fig. 2f). Similar binding of both head regions was also observed for FAK, a

known talin interactor used here as a control (Fig. 2f). Further mapping identified the binding site for PtdInsPKI γ -90 within the third lobe (L3) of the clover-shaped FERM domain of talin (Fig. 2d, g); that is, in the subdomain that harbours structural similarity with plekstrin homology (PH), phosphotyrosine-binding (PTB) and enabled/vasp homology 1 (EVH1) domains^{16,17}.

Affinity chromatography of brain extracts on various fragments of the 28aa-tail mapped the minimal region of PtdInsPKI γ -90 responsible for talin binding to the amino acid sequence WVYSP (Fig. 2h). To define further the characteristics of this interaction, we screened a phage display random peptide library with a GST fusion of the head domain of talin 2. Positive phages were confirmed using

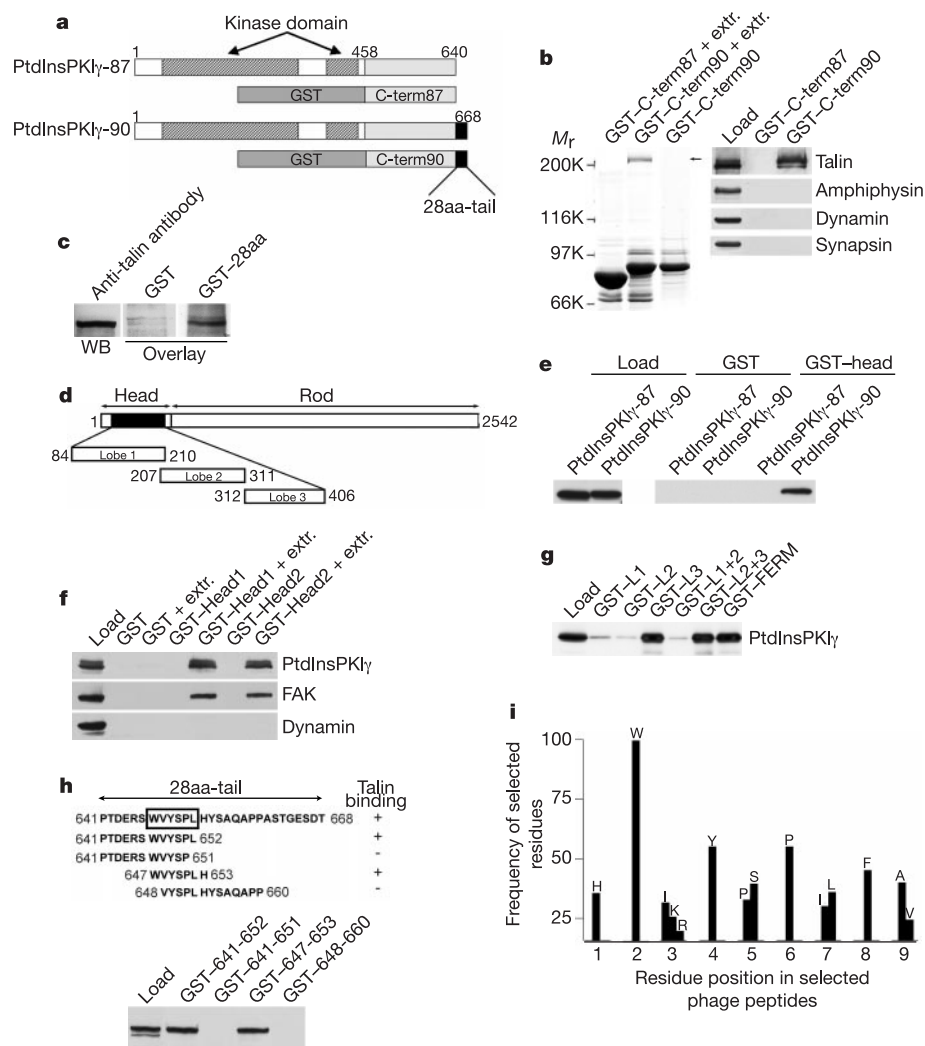


Figure 2 Interaction of PtdInsPKI γ -90, via a short amino acid sequence present in its 28aa-tail, with the FERM domain of talin. **a**, Domain of GST–PtdInsPKI γ -87 and GST–PtdInsPKI γ -90. **b**, Affinity chromatography of a rat brain extract (extr.) on GST fusions of the C-terminal domains of PtdInsPKI γ -87 and PtdInsPKI γ -90. Left, Coomassie-blue-stained SDS–polyacrylamide gel electrophoresis (PAGE) of the eluate. The arrow points to a 240K band that was identified by MALDI–TOF as talin 2. Right, Western blot of the same material showing the binding of talin, but not of control proteins, to the C-terminal domain of PtdInsPKI γ -90. **c**, Overlay assay showing direct binding of GST–28aa-tail, but not of GST alone, to talin. **d**, Diagram of human talin 2. The head contains the FERM domain, which consists of three lobes^{16,17} (black box). **e**, Talin 2 binds PtdInsPKI γ -90 via the head domain. *In vitro* translated HA-tagged PtdInsPKI γ -87 and PtdInsPKI γ -90 were affinity purified on a GST fusion of the head of talin 2, or on GST alone as a control. Anti-HA Western blot reveals the binding of PtdInsPKI γ -90. **f**, PtdInsPKI γ -90 binds to the heads of both talin 1 and talin 2. Western blot of the material affinity purified from a rat brain extract

by GST fusion proteins of the two head regions. Note the binding of PtdInsPKI γ -90 and of FAK (positive control), but not of dynamin (negative control) to both head regions.

g, Mapping of the PtdInsPKI γ -90-binding region of talin 2 to the third lobe (or subdomain C) of its FERM domain. A rat brain extract was affinity purified on GST fusions of the FERM domain or of its three lobes (L1, L2 and L3). An anti-PtdInsPKI γ -90 Western blot of the affinity purified material is shown. **h**, Mapping of the talin-binding region in the 28aa-tail of PtdInsPKI γ -90 by deletion analysis. The amino acid sequences of the 28aa-tail indicated in the top panel were fused to GST and used for affinity chromatography of a rat brain extract. The minimal region required for binding is boxed. An anti-talin Western blot of the affinity purified material is shown in the bottom panel. **i**, Graph showing results from a random peptide phage library screen with the head domain of talin 2. Amino acids occurring in more than 20% of selected peptides for each position are shown (see also Supplementary Information).

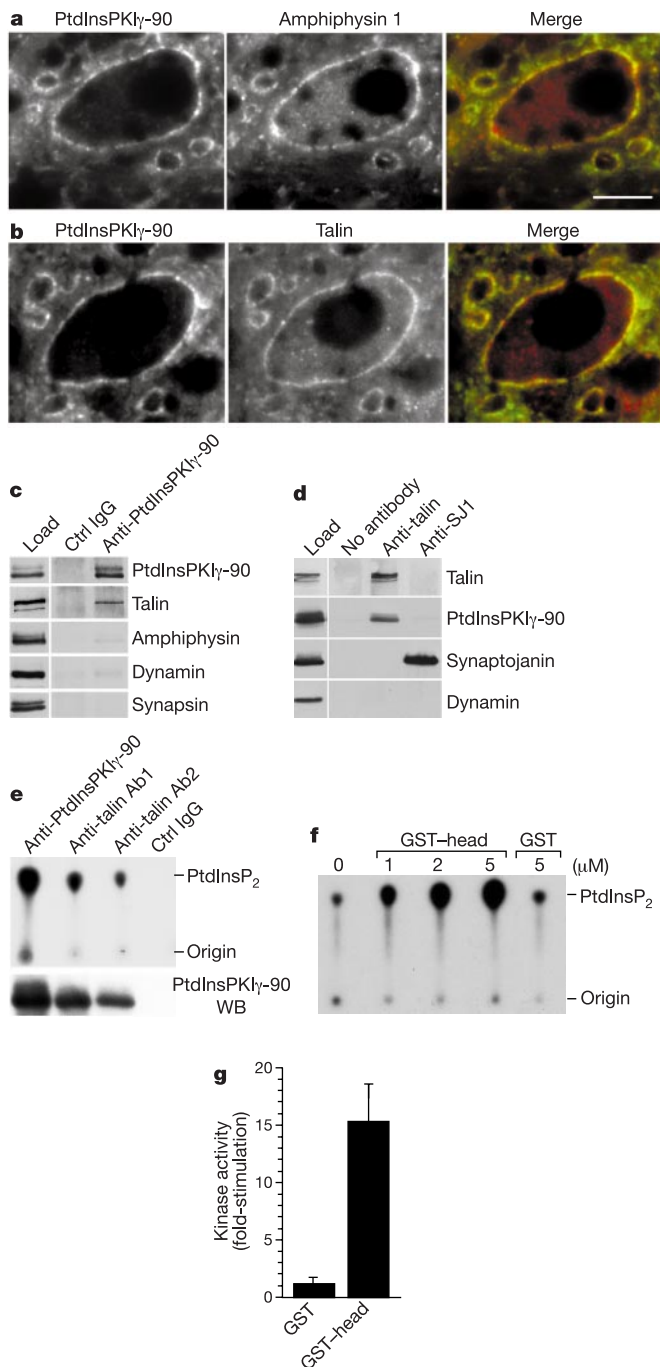


Figure 3 The interaction between talin and PtdInsPKI γ -90 occurs physiologically in brain. **a**, Double immunofluorescence of a rat brain section for PtdInsPKI γ -90 (anti-28aa-tail antibody, green) and for the synaptic marker amphiphysin 1 (red). **b**, Double immunofluorescence of the same brain area showing partial co-localization at synapses of talin (red) and PtdInsPKI γ -90 (green) immunoreactivity (anti-28aa-tail antibody). A motor neuron and some cross-sectioned dendrites surrounded by synapses are shown. Scale bar, 10 μ m. **c**, Western blot showing that an anti-PtdInsPKI γ -90 antiserum co-precipitates talin, but not other synaptic control proteins, from a rat brain extract. **d**, Western blot showing co-precipitation of PtdInsPKI γ -90 with an anti-talin antibody. Neither talin, nor PtdInsPKI γ -90 is present in a control immunoprecipitate generated by anti-synaptotagmin 1 antibodies. **e**, Thin-layer chromatography (TLC) from a lipid kinase assay demonstrating the presence of PtdInsP₂-synthesizing activity in two independent anti-talin immunoprecipitates (top). Anti-PtdInsPKI γ -90 and control IgG immunoprecipitations were used as positive and negative controls, respectively. PtdInsPKI γ -90 immunoreactivity recovered in anti-PtdInsPKI γ -90 and anti-talin immunoprecipitates (bottom). **f**, Effect of the head of talin 2 on the kinase activity of PtdInsPKI γ -90 *in vitro*. **g**, Quantification of the stimulating effect of 5 μ M GST-head ($n = 3$). GST was used as a control.

the third lobe of the FERM domain. Analysis of selected peptide sequences defined the consensus (Θ +/)W(Ψ +/)YXP Ψ Θ Ψ (where Θ , Ψ , X and + represent aromatic, aliphatic, any residue, and basic residues, respectively), which comprises the amino acid sequence present in PtdInsPKI γ -90 (Fig. 2i and Supplementary Information). The tryptophane residue at position +2 was present in all the peptides selected. Mutation of this residue in PtdInsPKI γ -90 (W647F) completely abolished binding of PtdInsPKI γ -90 to talin (data not shown). Notably, the talin-binding sequence of several integrin β -tails, which interact with the third lobe of talin's FERM domain through a conserved NPX(Y/F) motif¹⁸, was poorly represented in our selected peptides and only partially matches our consensus. The recent finding that integrin β -tails, unlike PtdInsPKI γ -90, also interact with the second lobe of talin's FERM domain¹⁸ suggests at least partially distinct binding sites.

Talin is an important component of focal adhesion plaques that links integrin to the actin cytoskeleton^{7,19}. Thus, the identification of talin as an interactor of PtdInsPKI γ -90 fits with the subcellular targeting of transfected GFP-PtdInsPKI γ -90 in fibroblasts or of endogenous PtdInsPKI γ in astrocytes. We next investigated whether the PtdInsPKI γ -90–talin interaction may occur also in neurons, and potentially contribute to the recruitment of PtdInsPKI γ to synaptic sites, where integrins²⁰ and other focal adhesion plaque components^{21–23} have been localized. An antibody directed to the 28aa-tail was generated and used together with an antibody that recognizes both talin 1 and 2 to compare, by immunofluorescence, the distribution of the two antigens in frozen sections of rat brain stem. Immunoreactivity for the 28aa-tail (and therefore for PtdInsPKI γ -90) partially co-localized with a pool of talin at a variety of synapses (Fig. 3a, b), consistent with their interaction. Furthermore, a substantial amount of talin, but not of control synaptic proteins, was detected in PtdInsPKI γ -90 immunoprecipitates (Fig. 3c). Conversely, a PtdInsPKI γ -90 immunoreactive band and PtdIns(4,5)P₂-synthesizing activity was specifically co-precipitated by talin antibodies (Fig. 3d, e).

Talin not only binds, but also regulates, PtdInsPKI γ -90. A GST fusion protein of the head of talin 2 (GST-head) markedly stimulated, in a dose-dependent fashion, the kinase activity of recombinant PtdInsPKI γ -90 (Fig. 3f). A 15-fold increase in the activity was induced by 5 μ M GST-head, whereas GST alone had no effect (Fig. 3g). This stimulatory effect was abolished by pretreating the talin head with a short peptide containing the talin-binding site of PtdInsPKI γ -90 (data not shown).

The localization of talin in the central nervous system has not been detected previously. The high similarity of talin 1 and 2 (ref. 24) (see also Supplementary Information) prevented us, so far, from analysing separately the two proteins with isoform-specific antibodies. However, the selective identification of talin 2 peptides in the rat brain material retained by immobilized 28aa-tail (see above), and the presence in brain of only low levels of talin 1 messenger RNA relative to other tissues²⁴, raises the possibility that talin in the brain may be represented primarily by talin 2. Similarly, PtdInsPKI γ -90 may be the predominant isoform of PtdInsPKI γ expressed in brain, as polymerase chain reaction with reverse transcription (RT-PCR) from brain complementary DNA detected mRNA from PtdInsPKI γ -90 only (data not shown). Furthermore, in western blots, several PtdInsPKI γ immunoreactive bands were labelled by the anti-28aa-tail-specific antibody and collapsed to a single band after *in vitro* dephosphorylation⁶ (data not shown).

Previous studies have emphasized the selective expression of PtdInsPKI γ in the nervous system^{5,6}. To determine whether our findings reflect an interaction that can occur in all cells, the distribution of immunoreactivity recognized by the 28aa-tail-specific antibody was investigated in non-neuronal cells and tissues. Reactivity, as determined by immunofluorescence, was detected at sites of focal adhesion in a variety of non-neuronal cell types tested, including NIH 3T3 fibroblasts (see Supplementary Information),

C2C12 cells, and BHK (data not shown) cells, where it partially co-localized with talin. By western blotting, immunoreactive bands that were in the size range of brain PtdInsPKI γ -90 and that could be affinity-purified by the immobilized head of talin were observed in all of several non-neuronal tissues tested, although at much lower

concentrations than in brain (see Supplementary Information).

Talin-mediated recruitment and regulation of PtdInsPKI γ -90 may represent a mechanism mediating for the local production of PtdIns(4,5)P₂ at cell adhesion sites, such as synapses and focal adhesion plaques. Of note, talin may also be an important effector of PtdIns(4,5)P₂, as this phospholipid regulates both its targeting to focal adhesion sites and its affinity for the cytoplasmic domain of β -integrin²⁵. In addition to talin, a variety of focal adhesion proteins, such as vinculin, α -actinin and several actin-binding proteins, interact with, and are regulated by, PtdIns(4,5)P₂ (ref. 9). The engagement of integrins in cell adhesion correlates with increased production of PtdIns(4,5)P₂ (ref. 26). Thus, both the attachment of the cell to the substrate and the anchoring of stress fibres to focal adhesion sites seem to be critically controlled by PtdIns(4,5)P₂.

To determine whether PtdInsPKI γ -90 can affect focal adhesion formation and/or stabilization, we investigated the effect of its overexpression as a GFP-tagged protein in NIH 3T3 cells. Transfection of both PtdInsPKI γ -87 and PtdInsPKI γ -90 greatly increased the total PtdIns kinase activity of fibroblast extracts relative to transfection of GFP alone (Fig. 4a). However, the activity associated with anti-talin immunoprecipitates was markedly enhanced only on PtdInsPKI γ -90 transfection, suggesting a high local production of PtdIns(4,5)P₂ in proximity to talin-containing complexes in these cells (Fig. 4a). We next examined the effect of these transfections on focal adhesions as assessed by immunostaining for FAK (Fig. 4b) and vinculin (data not shown). Most of the cells transfected with GFP alone or with PtdInsPKI γ -87 adopted a well-spread morphology and exhibited a large number of focal adhesion plaques (Fig. 4b) and stress fibres (data not shown). Similarly, two other PtdInsPKI isoforms, α and β , were not targeted to focal adhesions (data not shown) and accordingly, did not affect cell spreading (Fig. 4c). In contrast, most cells expressing high levels of PtdInsPKI γ -90, but not cells expressing a talin-binding-deficient mutant of PtdInsPKI γ -90 (W647F), were rounded and loosely attached to the substrate, with only few adhesion sites (Fig. 4b, c). Finally, expression of the entire C-terminal region of PtdInsPKI γ -90, which lacks the kinase domain, had an intermediate level of effect, possibly due to competition with endogenous PtdInsPKI γ -90 for talin binding (Fig. 4c). Interestingly, the activation loop of PtdInsPKI γ -90, which contributes to plasma membrane targeting of type I PtdIns kinases and determines their substrate specificity²⁷, was required for this effect (data not shown). Accordingly, expression of the 28aa-tail had no obvious effect on adhesion (data not shown).

The property of talin to recruit and activate PtdInsPKI γ -90, together with the proposed regulatory role of PtdIns(4,5)P₂ on talin's function²⁵, point to the existence of positive feedback mechanisms that regulate critical aspects of cell adhesion. A likely interpretation of our transfection data is that a perturbation of the talin–PtdInsPKI γ -90 interaction disrupts cell adhesion by affecting normal PtdIns(4,5)P₂ turnover in the lipid environment surrounding talin. In conclusion, we have identified a protein–protein interaction that probably has a critical role in the control of cell adhesion. This interaction occurs also at synapses, which are highly specialized cell adhesion sites. Additional factors such as small GTPases probably cooperate with talin in the recruitment and regulation of PtdInsPKI γ -90 (ref. 28). Further studies of these interactions may reveal not only new aspects of cell–substrate adhesion, but also new mechanisms in synapse development and function. □

Methods

Cloning of talin 2

Peptide sequencing, MALDI and quantitative TOF mass spectrometry were performed by the Keck Research Facility at Yale University (<http://www.info.med.yale.edu/wmkeck/prochem/>). The partial human clone KIAA0320 and mouse clone AK017597 were found to contain a significant number of exact peptide sequence matches and to encode talin 2

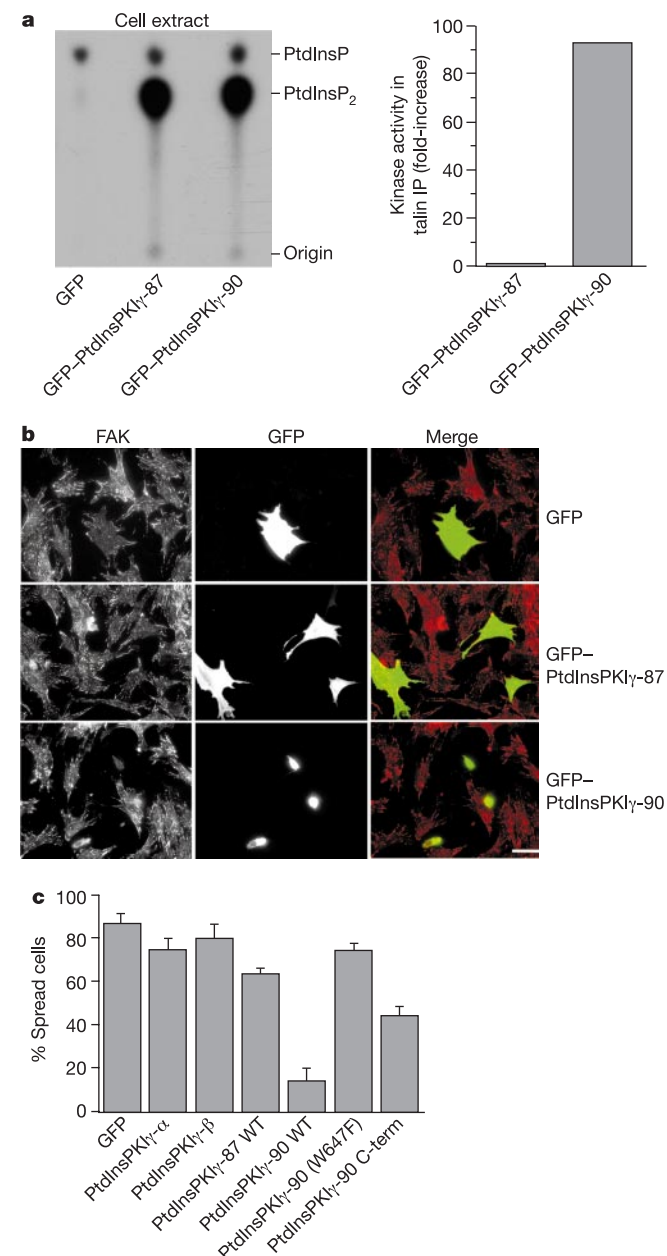


Figure 4 Disruption of normal phosphoinositide metabolism at focal adhesion plaques interferes with cell adhesion in NIH 3T3 cells. **a**, TLC demonstrating a marked increase of PtdIns phosphate kinase activity associated with extracts from fibroblasts transfected with either GFP–PtdInsPKI γ -87 or GFP–PtdInsPKI γ -90, but not with GFP alone (left panel). In contrast, anti-talin immunoprecipitates from cells transfected with GFP–PtdInsPKI γ -90, but not with GFP–PtdInsPKI γ -87, contained greatly enhanced activity (right panel). The graph shows the increase in kinase activity relative to GFP-transfected cells. **b**, High level expression of GFP–PtdInsPKI γ -90, but not of GFP–PtdInsPKI γ -87 or of GFP alone, disrupts focal adhesion, as shown by the round shape of the cells and by a counterstaining with anti-FAK antibodies. Scale bar = 15 μ m. **c**, Quantification of the percentage of transfected cells that have spread morphology and containing focal adhesion plaques, based on anti-FAK counterstaining. Cells were transfected with the constructs indicated, which were all GFP-tagged, except for PtdInsPKI α and - β , which were HA-tagged. Values denote means $\pm$ s.d. ($n = 3$).

(see Supplementary Information). Full-length talin 2 clones were PCR-amplified from human brain and skeletal muscle Marathon-Ready cDNA libraries (BD Biosciences Clontech).

Antibodies and constructs

A rabbit serum specific for PtdInsPKI γ -90 was raised against a peptide corresponding to the 28aa-tail (amino acids 635–662 of mouse PtdInsPKI γ -90). The following antibodies were from commercial sources: anti-talin clone 8d4 (Sigma), antiserum C-20 (Santa Cruz Biotechnology) and clone TA205 (Chemicon); anti-vinculin (Sigma) and anti-FAK C-20 (Santa Cruz Biotechnology). Other antibodies used in this study are described elsewhere⁶. The C-terminal domains of human PtdInsPKI γ -87 (amino acids 459–640) and PtdInsPKI γ -90 (amino acids 458–668) were PCR-amplified from clone KIAA0589 and subcloned into pGEX-6P-1 vector (Amersham-Pharmacia). Deletion constructs were generated in the same vector. For expression of GFP fusion proteins, human PtdInsPKI γ -87, -90, 28aa-tail (amino acids 641–668) and PtdInsPKI γ -90 C-terminal (amino acids 384–668) were subcloned into the pEGFP2C vector (BD Biosciences Clontech). The head of human talin 2 and mouse talin 1, together with fragments from the FERM domain of talin 2, were subcloned into pGex4T1. Recombinant PtdInsPKI γ -90 was produced in insect cells using the baculovirus system (BD Pharmingen).

Biochemical procedures

Affinity chromatography experiments and immunoprecipitations were performed from rat brain detergent extract as described previously^{6,29}. For the lipid kinase assay, immunoprecipitates were incubated for 10 min at 37 °C in 40 μ l kinase buffer (25 mM HEPES pH 7.4, 100 mM KCl, 1 mM EGTA, 2.5 mM MgCl₂) containing 20 μ g phosphoinositides (Sigma), 20 μ Ci [γ -³²P]ATP and 50 μ M cold ATP. Samples were then processed as previously described⁶. For kinase assays involving recombinant PtdInsPKI γ -90, 25 ng enzyme was used. Kinase assays performed with fibroblasts used 50 μ g extract. The blot overlay assay with GST and GST-28aa-tail was performed as described²⁹. The phage display library screening was performed according to ref. 30.

Cell transfection and immunofluorescence

NIH 3T3 cells were grown and maintained on plastic culture plates at 37 °C in a 5% CO₂ atmosphere in DMEM containing 10% fetal bovine serum. Cells were detached with PBS supplemented with 0.05% trypsin, 1 mM EDTA, replated on fibronectin-coated glass coverslips and transfected using LipofectAmine reagent (Life technologies). Twenty-four hours later, cells were fixed, immunostained and analysed by either confocal or epifluorescence microscopy. For the quantification of spread cells, 150–200 cells were scored from each of three independent experiments.

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Type I γ phosphatidylinositol phosphate kinase targets and regulates focal adhesions

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The ability of cells to form cell contacts, adhere to the extracellular matrix, change morphology, and migrate is essential for development, wound healing, metastasis, cell survival and the immune response. These events depend on the binding of integrin to the extracellular matrix, and assembly of focal adhesions, which are complexes comprising scaffolding and signalling proteins organized by adhesion to the extracellular matrix^{1–3}. Phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) regulates interactions between these proteins, including the interaction of vinculin with actin and talin^{4–9}. The binding of talin to β -integrin is strengthened by PtdIns(4,5)P₂, suggesting that the basis of focal adhesion assembly is regulated by this lipid mediator^{9,10}. Here we show that the type I phosphatidylinositol phosphate kinase isoform- γ 661 (PIPKI γ 661), an enzyme that makes PtdIns(4,5)P₂, is targeted to focal adhesions by an association with talin. PIPKI γ 661 is tyrosine phosphorylated by focal adhesion associated kinase signalling, increasing both the activity of phosphatidylinositol phosphate kinase and its

association with talin. This defines a mechanism for spatial generation of PtdIns(4,5)P₂ at focal adhesions.

Adherence to extracellular matrix stimulates the generation of PtdIns(4,5)P₂ (refs 4, 6) and potentially modulates focal adhesion assembly around clusters of integrins^{1–10}. Type I phosphatidylinositol phosphate kinase isoforms (PIPKIs) generate the lipid messenger PtdIns(4,5)P₂ (refs 11, 12). The PIPKI γ transcripts are alternatively messenger RNA spliced, resulting in the PIPKI γ 635 and PIPKI γ 661 isoforms that differ by a 26-amino-acid carboxy-terminal extension¹³.

PIPKI γ 661 is specifically targeted to focal adhesions (Fig. 1a). To determine if the C-terminal 26 amino acids are sufficient for targeting, these residues were fused to green fluorescent protein, GFP. The GFP γ 26 is poorly targeted to focal adhesions, suggesting that other regions within PIPKI γ 661 are also required. To define these constraints, we prepared chimaeras using PIPKI α , an enzyme not targeted to focal adhesions (see Supplementary Information). The PIPKI α/γ chimaeras contained 178 residues from the C terminus of PIPKI γ 661 (PIPKI α/γ 661) or simply the PIPKI γ 661 26 amino acids fused to the C terminus of PIPKI α (PIPKI α/γ 26). PIPKI α/γ 661 and PIPKI α/γ 26 target to focal adhesions, but not PIPKI α/γ 635 (Fig. 1a). Enhanced targeting of the PIPKI α constructs with the C-terminal 26 residues reflects structural or functional features of the phosphatidylinositol phosphate kinases, such as dimer formation, which is required for activity at membranes^{12,14}.

We used two approaches to identify proteins that specifically interact with PIPKI γ 661. Haemagglutinin (HA)-tagged PIPKI γ 661 or PIPKI γ 635 were expressed in HEK 293T cells, and isolated by immunoprecipitation. As numerous focal adhesion proteins are tyrosine phosphorylated, the immunoprecipitates were immunoblotted with anti-phosphotyrosine antibodies. A tyrosine-phosphorylated protein of relative molecular mass 230,000 (M_r 230K) was selectively immunoprecipitated with PIPKI γ 661 (Supplementary Information). This protein was identified as talin^{15,16} (Fig. 1b). Vinculin, focal adhesion associated kinase (FAK) and tensin did not interact with PIPKI γ 661 (Supplementary Information). Only the PIPKI α/γ chimaeras that target to focal adhesions associated with talin (Fig. 1b). To confirm the talin–PIPKI γ 661 interaction, talin was immunoprecipitated from HEK 293T cells overexpressing PIPKI γ 661 or PIPKI γ 635. Talin pulled down PIPKI γ 661, but not PIPKI γ 635 (Fig. 1b). In addition, endogenous PIPKI γ splice isoforms are widely expressed in various cell lines. When endogenous PIPKI γ was immunoprecipitated from a variety of cells types, talin association was observed (Supplementary Information).

A yeast two-hybrid screen using the 178-residue C-terminal region of PIPKI γ 661 as bait resulted in the isolation of 12 talin clones. This demonstrated a direct interaction, and narrowed the interacting region to residues 150 to 480 in the talin head region. This region encompasses a portion of F1 and the F2 and F3 regions of talin's 'band 4.1, ezrin, radixin, moesin' (FERM) homology domain. The talin FERM domain also contains regions shown to interact with other focal adhesion proteins^{17–20} (Fig. 1c).

These data show that the PIPKI γ 661–talin association is important for targeting PIPKI γ 661 to focal adhesions. This is reinforced by the immunofluorescent colocalization of PIPKI γ 661 and talin in NRK cells (Fig. 2). As PtdIns(4,5)P₂ modulates the association of talin with β -integrin and other focal adhesion proteins^{7,9,10}, the PIPKI γ 661–talin interaction may regulate the focal adhesion assembly of talin. Moderate overexpression of PIPKI γ 661 resulted in substantially larger talin-containing focal adhesions in 43% of cells compared to non-transfected cells (Fig. 2). This supports a model where PIPKI γ 661 modulates talin assembly into focal adhesions. High overexpression of PIPKI γ 661, but not PIPKI γ 635, resulted in a loss of talin from focal adhesions in 49% of the transfected cells (Fig. 2). These data indicate that increasing levels of PtdIns(4,5)P₂ at focal adhesions cause talin assembly and disassembly, suggesting a dynamic model. Overexpression of

PIPKI isoforms reorganizes actin from stress fibres to cortical actin filaments^{11,13}. Thus, phenotypes observed by high overexpression of PIPKI γ 661 may also affect focal adhesions indirectly.

Kinase inactive mutants, when expressed in cells, will often disrupt the function of the wild-type kinase by competing for

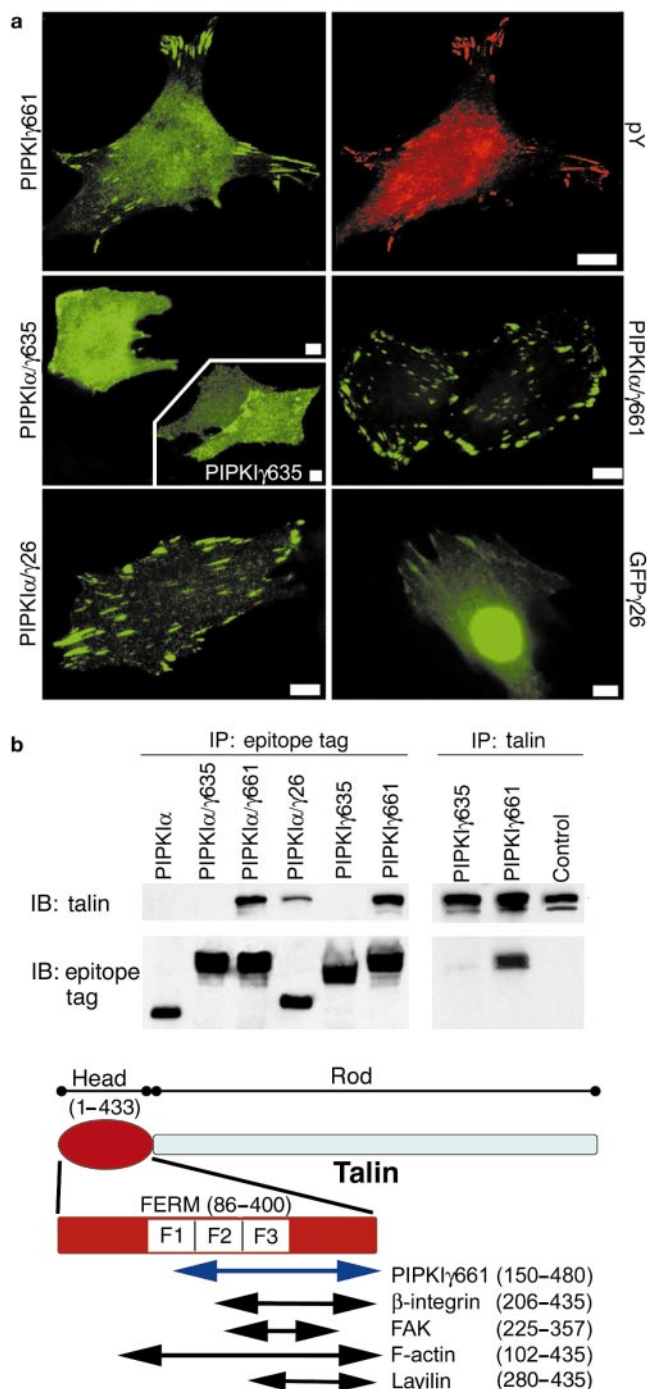


Figure 1 PIPKI γ 661 is targeted to focal adhesions by an association with talin. **a**, Top, colocalization of HA-PIPKI γ 661 and focal adhesions in NRK cells stained with anti-HA (green) and polyclonal anti-phosphotyrosine (pY) (red) antibodies. Fusion proteins containing the C-terminal 26 amino acids from PIPKI γ 661 show targeting to focal adhesions in NRK cells. c-Myc-PIPKI α/γ chimaeras and HA-PIPKI γ 635 were stained with c-Myc and HA antibodies, respectively. See Supplementary Information for generation of chimaeras. Scale bar, 10 μ m. **b**, Overexpressed PIPKI constructs were immunoprecipitated (IP) from HEK 293T cells and then immunoblotted (IB) with indicated antibodies. **c**, PIPKI γ 661 interacts with talin in the FERM domain as determined by yeast two-hybrid screen. Illustration of other talin binding proteins in FERM domain.

targeting or regulation. Expression of a kinase-inactive ('kinase-dead') PIPKI γ 661 mutant (PIPKI γ 661kd) resulted in a loss of talin targeting to focal adhesions in 70% of the cells expressing high levels (Fig. 2). This is consistent with the proposed role for PtdIns(4,5)P₂ in talin assembly into focal adhesions¹⁻⁹.

During adhesion and spreading, cells assemble focal adhesions rapidly, providing a good model to define the role of PIPKI γ 661 in membrane and focal adhesion assembly of talin. PIPKI γ 661 facilitated the targeting of talin to the plasma membrane in adhering cells (Fig. 3). This was independent of PIP kinase activity, as PIPKI γ 661kd also facilitated targeting (Fig. 3 inset; see also Supplementary Information). Significantly, PIPKI γ 661 facilitated the assembly of talin-containing focal adhesions in spreading cells, and this was dependent on PIP kinase activity (Fig. 3). These data show that PIPKI γ 661 regulates both plasma membrane targeting and the efficient assembly of talin into focal adhesions.

The association between PIPKI γ 661 and talin was also regulated by adhesion to type I collagen. In Fig. 4a, we show co-immunoprecipitation of talin with PIPKI γ 661 from HEK 293T cells in suspension versus cells adherent to type I collagen. Talin strongly associated with PIPKI γ 661 in adherent cells, but not in suspended cells. However, in suspended cells the PIPKI γ 661 association with talin was restored when cells were stimulated with lysophosphatidic acid (LPA) or platelet-derived growth factor (PDGF).

Tyrosine phosphorylation of focal adhesion proteins is stimu-

lated by adhesion, and is important for focal adhesion assembly²¹. PIPKI γ 661 is tyrosine phosphorylated, and this was dependent upon PIP kinase activity (Fig. 4a). Tyrosine phosphorylation was also stimulated upon cell adhesion to type I collagen. FAK is an important tyrosine kinase at focal adhesions and is activated by LPA²². So to determine FAK's role in tyrosine phosphorylation of PIPKI γ 661, we coexpressed dominant active FAK (CD2FAK)²³ with each of the PIPKI γ isoforms. This induced a ~4-fold increase in tyrosine phosphorylation of PIPKI γ 661, but not PIPKI γ 635, illustrating that focal adhesion targeting is required for tyrosine phosphorylation of PIPKI γ 661. Tyrosine phosphorylation of PIPKI γ 661kd was also stimulated by coexpression of CD2FAK, although to a lesser extent. Coexpression of the dominant-negative FAK related non-kinase (FRNK), which blocks endogenous FAK activity, diminished tyrosine phosphorylation of PIPKI γ 661. FRNK also inhibited adhesion-stimulated PIPKI γ 661 tyrosine phosphorylation. The FAK-induced tyrosine phosphorylation of PIPKI γ 661 correlates with a substantial increase in its association with talin. Tyrosine phosphorylation of PIPKI γ 661 may facilitate association with talin via the phosphotyrosine-binding site in the talin head²⁰. Significantly, CD2FAK but not FRNK specifically stimulated the activity of PIPKI γ 661. These data indicate that FAK signalling stimulates both talin association and PIP kinase activity, resulting in localized generation of PtdIns(4,5)P₂ at focal adhesions (see Supplementary Information).

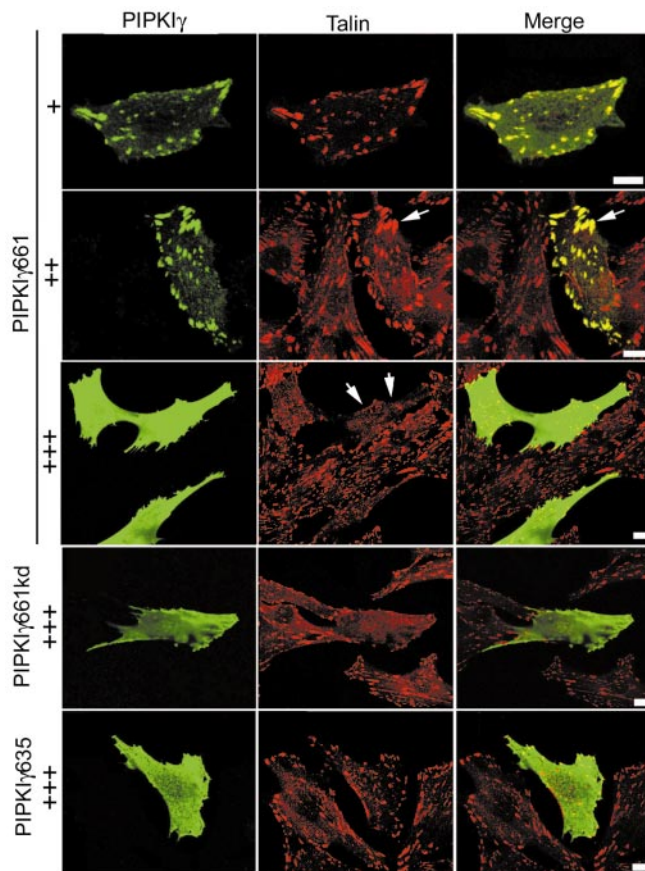


Figure 2 PIPKI γ 661 modulates assembly of talin into focal adhesions. Overexpressed PIPKI γ and endogenous talin in NRK cells were visualized by anti-PIPKI γ serum (green) and anti-talin antibody (red), respectively. Relative expression level of PIPKI γ is shown (+). Low HA-PIPKI γ 661 (+) colocalized with talin, and the increasing moderate expression (++) induced larger talin-containing focal adhesions, whereas high overexpression (+++) diminished talin assembly into focal adhesions. High expression of PIPKI γ 661kd abolished talin assembly into focal adhesions. High expression of PIPKI γ 635 constructs had no effect on focal adhesions. Scale bar, 10 μ m.

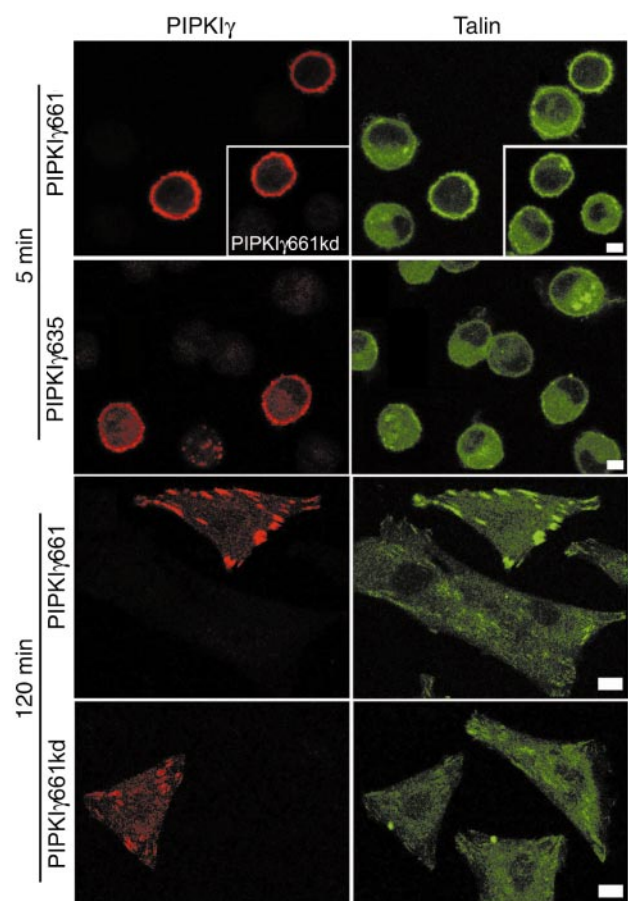


Figure 3 PIPKI γ 661 facilitates talin targeting to membranes and assembly into focal adhesions. Transfected NRK cells were suspended for 1 h, then replated on type I collagen (see Methods). After the indicated time, localization of PIPKI γ (green) and talin (red) in cells was visualized with anti-PIPKI γ serum and anti-talin antibodies, respectively. Inset, location of PIPKI γ 661kd and talin. See Supplementary Information for quantification of PIPKI γ and talin localization. Scale bar, 10 μ m.

A functional link between PIPKI γ 661 and FAK was reinforced by the observation that PIPKI γ 661kd but not PIPKI γ 661 disrupts targeting of FAK to focal adhesions. PIPKI γ 661 and PIPKI γ 661kd both target to focal adhesions and colocalize with FAK (Fig. 4b and

data not shown). High expression of PIPKI γ 661 did not affect FAK targeting. In contrast, the expression of PIPKI γ 661kd resulted in complete loss of FAK targeting to focal adhesions in 39% of the expressing cells. A number of other focal adhesion proteins were analysed to determine the effect of PIPKI γ 661 and PIPKI γ 661kd expression. From these studies, paxillin and vinculin were more resistant to changes induced by ectopic expression of PIPKI γ 661 or PIPKI γ 661kd (see Supplementary Information).

The interaction between PIPKI γ 661 and talin appears critical for focal adhesion assembly. This is supported by observations that a β -integrin binding site regulated by PtdIns(4,5)P₂ is located in the head region of talin within the FERM domain^{9,17–19}. Proteins containing the FERM domain, such as protein 4.1 and talin, interact with integral membrane proteins, such as glycophorin or integrins, and these interactions are modulated by PtdIns(4,5)P₂ (refs 9, 19, 24, 25). Therefore, the PIPKI γ 661–talin interaction may be important in the initiation of focal adhesion assembly via regulation of integrin binding and other focal adhesion proteins by PtdIns(4,5)P₂ (refs 1–3, 7–9).

We suggest that, upon clustering of integrins, talin and PIPKI γ 661 are recruited to focal adhesions, inducing synthesis of PtdIns(4,5)P₂. Spatial generation of PtdIns(4,5)P₂ facilitates the recruitment and regulation of proteins such as vinculin, α -actinin and FAK. FAK, in a manner that depends on PtdIns(4,5)P₂, tyrosine phosphorylates PIPKI γ 661, and further stimulates production of PtdIns(4,5)P₂. Regulated and localized generation of PtdIns(4,5)P₂ facilitates the assembly and/or disassembly of focal adhesions. PIPKI activity is also regulated by small G proteins^{4,6,26} and by phosphatidic acid, a product of phospholipase D (PLD)²⁷. PLD activity is required for actin stress fibre formation and α -actinin assembly at focal adhesions²⁸. In many cells, adhesion is essential for survival and growth, which require phosphoinositol 3-kinase (PI3K) activity²¹. PI3K is also modulated by FAK, and may be dependent upon PIPKI γ 661 for its substrate. Consequently, generation of PtdIns(4,5)P₂ by PIPKI γ 661 may also control the generation of messengers derived from PtdIns(4,5)P₂. This positions PIPKI γ 661 at a signalling branch point that modulates both focal adhesion assembly and signals emanating from focal adhesions. □

Methods

Constructs

Site-directed mutagenesis for creation of kinase-dead PIPKI γ 661 (D253A) was performed using PCR-primer overlap extension with mutagenic primers¹². The mutagenic primers were 5'-CAAAATGCACCTTAAGTT CGCCCTCAAGGGCTCCAC-3' and 5'-GTACGTG GAGCCCTTGAGGGCGGAA CTTAACCCTGC-3'. The flanking primers were 5'-CTATG CACCTGTTGCCTTCGCTACTTC-3' and 5'-GGCGTGAATACAGAGCCTTC-3'. The mutation was confirmed by DNA sequence analysis. A 0.5-kilobase *Xma*I–*Cl*aI restriction fragment was used to replace the corresponding restriction fragment in the HA–PIPKI γ 661/pcDNA3 construct. See Supplementary Information for generation of GFP constructs, c-Myc–PIPKI α/γ chimaeras, and recombinant protein expression vectors.

Yeast two-hybrid screen

The yeast two-hybrid screen²⁹ was performed in the Molecular Interaction Facility at the University of Wisconsin according to their protocols. Libraries screened were: mouse embryonic, human B cell, human breast, human prostate, human placenta and mouse brain. See <http://www.biotech.wisc.edu/mif/Lipid>

Kinase assays

Activity of purified recombinant or immunoprecipitated PIPKI proteins was assayed against 25 μ M P14P micelles as previously described¹².

Cell culture, transfection, immunofluorescence and confocal microscopy

HEK 293T cells and NRK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc.) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics. NRK cells were transfected using FuGENE 6 (Roche) according to manufacturer's instructions for 24 h. HEK 293T cells in 1 μ g ml⁻¹ collagen-coated dishes were transfected using 2 μ g PIP kinase expression vector together with 3 μ g empty pcDNA3 vector, CD2FAK, or FRNK as indicated, using the calcium phosphate–DNA coprecipitation method for 48 h.

Immunofluorescence and confocal microscopy were performed as described¹². Z series were created by sequentially scanning green and red channels at 0.2 μ m steps. Single sections were exported to Adobe Photoshop 6.0 for final image processing.

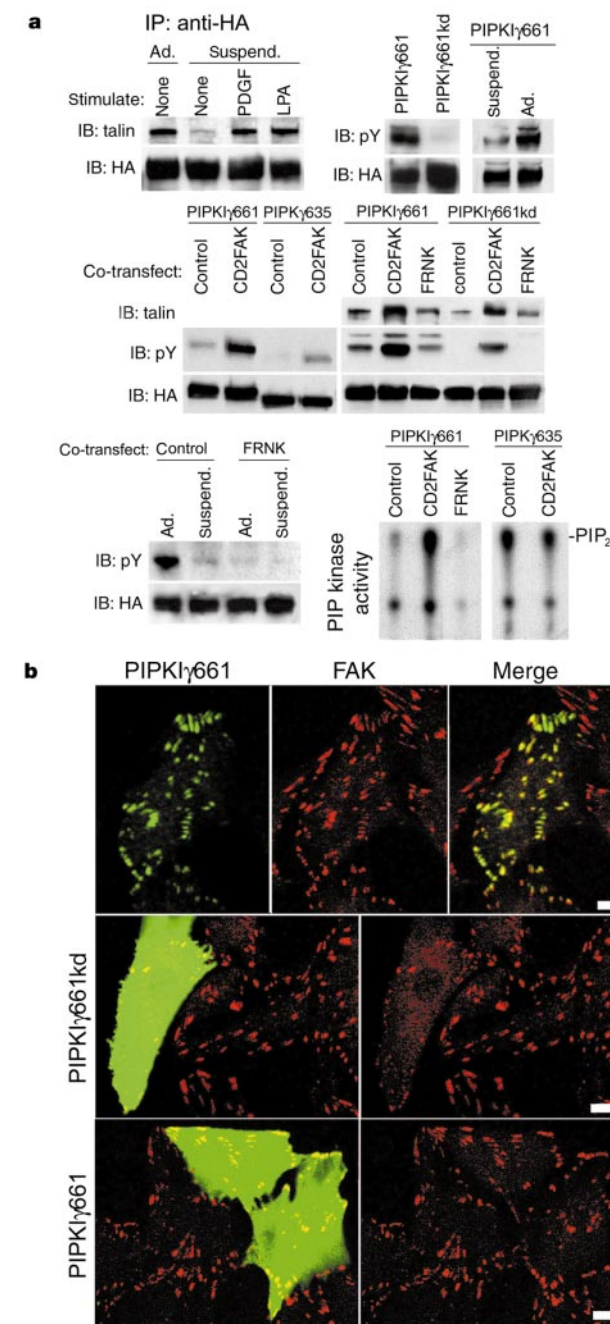


Figure 4 Functional linkage between PIPKI γ 661 and FAK signalling. **a**, In all experiments, HA–PIPKI γ was immunoprecipitated (IP) from HEK 293T cells with anti-HA antibody and analysed by immunoblotting (IB) or kinase assay as indicated. Top left, PIPKI γ 661–talin association in adherent (ad.), suspended (suspend.), or suspended cells stimulated as indicated. Top right, PIPKI γ 661, but not PIPKI γ 661kd, is tyrosine phosphorylated, which is dependent upon adhesion. Middle, phosphorylation of PIPKI γ and talin association were shown when cotransfected with CD2FAK or FRNK. Bottom left, adhesion-dependent PIPKI γ 661 phosphorylation was inhibited by FRNK. Bottom right, PIPKI γ activity when coexpressed with indicated proteins. (See Supplementary Information for quantification.) **b**, High expression of PIPKI γ 661kd but not PIPKI γ 661 (visualized by anti-PIPKI γ serum, green) abolishes FAK (visualized with anti-FAK, red) from focal adhesions in NRK cells. Scale bar, 10 μ m. N ≥ 4 for all results in this Letter.

Expression and purification of PIP kinase in *Escherichia coli*

His-tagged PIPK α , PIPK γ 661 and C-tail of PIPK γ 661 were expressed in *E. coli* and purified as described²⁷.

Antibodies

Anti-talin, anti-vinculin and anti-Flag (M2) antibodies are from Sigma. Monoclonal mouse anti-PY (4G10), anti-FAK (4.47) and anti-paxillin (5H11) are from Upstate. Polyclonal rabbit anti-PY antibody is from Transduction Laboratories. Anti-HA and anti-c-Myc antibodies are from Covance. Polyclonal PIPK γ anti-serum was generated by Covance using purified His-tagged PIPK γ 661. Secondary antibodies are from Jackson ImmunoResearch. Anti-serum was purified on an affinity column generated by coupling recombinant C-terminus of PIPK γ 661 to cyanogen bromide-activated Sepharose 4B (Sigma) as described³⁰.

Immunoprecipitation and immunoblotting

Immunoprecipitation was performed as described³¹. Briefly, transfected HEK 293T cells were harvested and lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 5.0 mM NaF, 2 mM Na₃VO₄, 4 mM Na₂P₂O₇, 1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 10% glycerol, then centrifuged and incubated with protein A-Sepharose and 5 μ g anti-HA, anti-c-Myc, anti-Flag, or anti-talin antibody as indicated at 4 °C overnight. The immunocomplexes were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and analysed as indicated. For kinase assay, the immunocomplexes were washed by and stored in kinase buffer.

Cell stimulation and spreading

Transfected HEK 293T cells were washed with PBS and detached with 0.25% trypsin at 37 °C. The cells were harvested and washed with PBS, and then suspended in serum-free DMEM at 37 °C for 1 h. After stimulation with 10 ng ml⁻¹ platelet-derived growth factor (PDGF) (R&D Systems Inc.) or 1 μ M LPA (Sigma) or serum at 37 °C for 15 min, cells were lysed as above for immunoprecipitation. For spreading assays, transfected NRK cells were detached with 1 mM EDTA, washed with PBS, then 0.1 $\times$ 10⁶ cells were plated on 1 μ g ml⁻¹ collagen-coated coverslips in serum-free DMEM. After the indicated time, the coverslips were fixed and stained as described above.

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Nbs1 is essential for DNA repair by homologous recombination in higher vertebrate cells

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Double-strand breaks occur during DNA replication and are also induced by ionizing radiation. There are at least two pathways which can repair such breaks: non-homologous end joining and homologous recombination (HR). Although these pathways are essentially independent of one another, it is possible that the proteins Mre11, Rad50 and Xrs2 are involved in both pathways in *Saccharomyces cerevisiae*¹. In vertebrate cells, little is known about the exact function of the Mre11–Rad50–Nbs1 complex in the repair of double-strand breaks because Mre11- and Rad50-

null mutations are lethal². Here we show that Nbs1 is essential for HR-mediated repair in higher vertebrate cells. The disruption of Nbs1 reduces gene conversion and sister chromatid exchanges, similar to other HR-deficient mutants³. In fact, a site-specific double-strand break repair assay showed a notable reduction of HR events following generation of such breaks in Nbs1-disrupted cells. The rare recombinants observed in the Nbs1-disrupted cells were frequently found to have aberrant structures, which possibly arise from unusual crossover events, suggesting that the Nbs1 complex might be required to process recombination intermediates.

The initial steps in the repair of double-strand breaks (DSBs) by HR involve the processing of DNA ends to produce 3' single-stranded tails which are the substrates for homologous pairing and strand invasion⁴. The Rad50-Mre11-Nbs1 complex has been suggested as a candidate which can generate the nuclease activity necessary to produce these tails⁵⁻¹⁰. Mre11 and Rad50 are highly conserved in *Schizosaccharomyces pombe*¹¹, mouse and human cells¹², whereas Xrs2 is not highly conserved. The known functional human homologue of Xrs2 is NBS1, which is mutated in Nijmegen breakage syndrome (NBS)¹³⁻¹⁵. NBS1 is reported to be a regulator of some of the biochemical activities of the NBS1/MRE11/RAD50 complex, such as ATP-driven DNA unwinding and nuclease activity⁸. However, little is known of the relationship between the non-homologous end joining (NHEJ) and HR repair pathways and the NBS1-MRE11-RAD50 complex, because in Mre11-null or Rad50-null vertebrate cells, spontaneous DNA break induction is lethal, in contrast to yeast mutants². As Nbs1-null mutations are lethal in embryonic mice¹⁶, only the creation of an Nbs1-null cell

line can make it possible to analyse the role of this complex in DNA repair pathways in mammalian cells.

To investigate the role of the Nbs1 complex in HR in vertebrate cells, we established an Nbs1 knockout cell line by using the hyper-recombinogenic chicken B-cell line DT40¹⁷. Exon 4 of the three Nbs1 alleles in DT40 cells was targeted (Fig. 1). The *Nbs1*^{-/-} cells are still viable, although they exhibit slow growth (Fig. 1e) owing to a prolonged cell cycle time (data not shown). Analysis by polymerase chain reaction with reverse transcription (RT-PCR) confirmed the absence of exons 1 to 6 (Fig. 1c) in Nbs1 transcripts, although all the cell lines express truncated NBS1 transcripts beginning from exon 7 (data not shown). In spite of this observation, there was no detectable expression of Nbs1 carboxy-terminal peptides using immunoblot analysis (Fig. 1d) in *Nbs1*^{-/-} cells, although such expression has recently been reported in NBS patients¹⁸. These data suggest that the *Nbs1*^{-/-} cells are likely to be Nbs1-null mutants. The *Nbs1*^{-/-} cell line exhibited hypersensitivity to ionizing radiation (Fig. 2a), and displayed an abnormal S-phase checkpoint behaviour called radio-resistant DNA synthesis (Fig. 2b). This behaviour is similar to that observed in cells from NBS patients

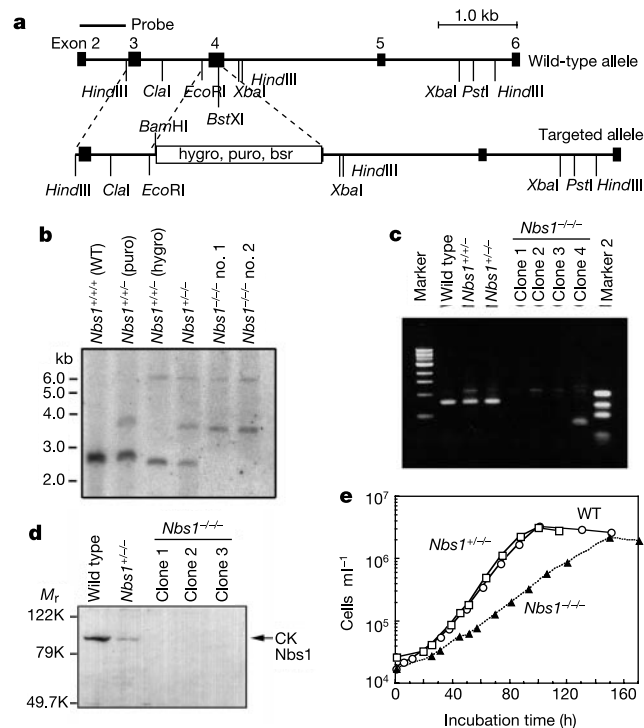


Figure 1 Generation of *Nbs1*^{-/-} clones. **a**, Schematic representation of a partial restriction map of the chicken *Nbs1* locus and the configuration of the targeted loci. **b**, Southern blot analysis. *Xba*I-digested genomic DNA was hybridized with the probe shown in **a**. **c**, RT-PCR analysis for wild type, heterozygous mutants, and homozygous mutants using a primer set which amplifies base pairs 39–820 of chicken *Nbs1* messenger RNA. Clone 4 was omitted from further analysis owing to a truncated transcript skipping exons 3–5. **d**, Western blot analysis of chicken Nbs1. *M_r*, relative molecular mass. **e**, The *Nbs1*^{-/-} cells display slow growth owing to their prolonged cell-cycle duration. WT, wild-type.

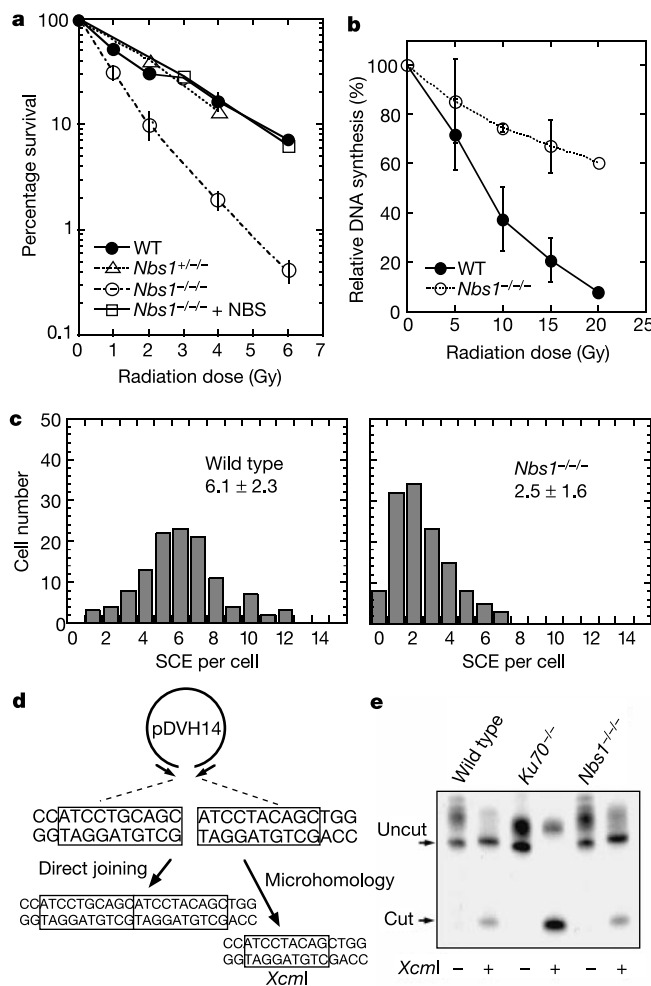


Figure 2 Cellular phenotypes of *Nbs1*^{-/-} cells. **a**, Increased radiation sensitivity of *Nbs1*^{-/-} cells and complementation by expression of human NBS1. **b**, Radiation-resistant DNA synthesis in *Nbs1*^{-/-} cells. **c**, Sister chromatid exchanges (SCEs) induced by mitomycin C (MMC) treatment. **d**, e, NHEJ is not affected in *Nbs1*^{-/-} cells. **d**, Outline of the end-joining assay. Joining of transfected linear DNA results in a *Xcm*I restriction site if the 10-bp microhomology is used to align the ends. **e**, End-joining in wild type, *Ku70*^{-/-} and *Nbs1*^{-/-} cells. Only the *Ku70*^{-/-} cells show increased microhomology use in this assay.

and in yeast with a deficiency in the Xrs2 complex^{19,20}. These cellular abnormalities are probably caused by the absence of a functional Nbs1 protein, because the cell's radiation sensitivity was complemented by the introduction of human NBS1 (Fig. 2a).

An increase of spontaneous- and radiation-induced chromosomal aberrations was also observed in *Nbs1*^{-/-} cells (Table 1). Most of the chromosomal aberrations were chromatid-type breaks and gaps (Table 1), even if the cells were irradiated during their G1 phase (7–8 h in wild type, and 7–12 h in *Nbs1*^{-/-}). This behaviour is similar to that seen in NBS patients, and the increase of chromatid-type breaks suggests that Nbs1 could be involved in HR repair. In support of this conclusion, *Nbs1*^{-/-} cells showed a marked reduction of sister chromatid exchanges after treatment with mitomycin C (Fig. 2c), which are known to depend on HR-mediated post-replicative repair³. The magnitude of this reduction is similar to that seen in HR-deficient cells such as Rad54 knockouts²¹. To test the involvement of Nbs1 in NHEJ, we performed end-joining assays using linearized plasmid DNA, which contains a 10-base-pair (10-bp) direct repeat at the ends. If this repeat is used to align the ends, the junction will sustain a 10-bp deletion, which creates an *XcmI* restriction site (Fig. 2d). The relative level of microhomology use at the junction is then determined by *XcmI* digestion of the amplified junction fragment. In wild-type DT40 cells, this results in 29% microhomology use, whereas the end-joining mutant *Ku70*^{-/-} cells use the microhomology in all of the junctions. *Nbs1*^{-/-} cells, on the other hand, do not show any increase in microhomology-directed joining (32%), showing that these cells do not have an end-joining defect such as that seen in *Ku70* mutants (Fig. 2e), although we cannot exclude a subtler defect. The absence of a profound end-joining defect was also supported by our observation that *Nbs1*^{-/-} cells were considerably more resistant to ionizing radiation than *Ku70*^{-/-} cells when irradiated at G1 phase (data not shown).

Next, the possibility of the involvement of Nbs1 in HR was tested by measuring targeted integration in the ovalbumin or immunoglobulin- λ loci in *Nbs1*^{-/-} cell lines. Targeted integration was completely suppressed in *Nbs1*^{-/-} cells, whereas about 40% of the drug-resistant transformants exhibited targeted recombination at specific loci in wild-type cells (Table 2). In contrast to radiation sensitivity, the human NBS1 gene was not able to fully complement the defect in targeted integration (Table 2). Because a slight decrease in interaction of human NBS1 with chicken Mre11 was observed (data not shown), it is possible that this difference might be caused

Table 2 Targeted integration frequencies

Genotype	Locus	Selection marker	Targeted integration/transformants analysed
Wild type	IgA	neo	10/24 (41.7%)
	OVA	Ecogpt	4/12 (33.3%)
<i>Nbs1</i> ^{-/-}	IgA	neo	0/38 (0%)
	OVA	Ecogpt	0/45 (0%)
		neo	0/34 (0%)
		hisD	0/24 (0%)
<i>Nbs1</i> ^{-/-} + human NBS1	OVA	Ecogpt	1/16 (6.2%)
		hisD	2/28 (7.1%)

In each experiment, 20 μ g of plasmids were transfected into $(0.6-1.0) \times 10^7$ cells. Then, transformants were randomly picked up and the target locus was analysed by Southern blotting. OVA represents ovalbumin. Selection markers are neo for neomycin (G418), Ecogpt (*E. coli* gpt for mycophenolic acid, and hisD for histidinol resistance).

by the lack of complete native function of human NBS1 in chicken cells. In any case, the result suggests that the ability of gene conversion to allow a targeted integration frequency of about 1/20, similar to levels seen in normal mammalian cells²², might be sufficient to provide normal radiation sensitivity. Based on knowledge of yeast HR, this result suggests that the absence of targeted integration in *Nbs1*^{-/-} cells might be due to their inability to process the DSB ends for initiation of HR events⁸, or due to the cell's inability to find the homologous sequence with Mre11²³.

Additional experiments were designed to look for evidence that the Nbs1 protein is related to the HR pathway. Wild-type or *Nbs1*^{-/-} cells were transfected with a modified SCneo reporter construct²⁴, and were observed for transient expression of the I-Sce I enzyme. In this system, a DSB is induced at the I-Sce I site in the S2neo sequence, and G418-resistant (neo-positive) cells survive if HR-mediated repair takes place successfully²⁴. The disruption of Nbs1 strongly reduced the frequency of neo-positive clones to background levels (measured with pBluescript transfected control cells) seen in wild-type cells (Table 3, *Nbs1*^{-/-} no. 13 and no. 17). In the SCneo-*Nbs1*^{-/-} no. 44 clone, no G418-resistance was detected even after expression of I-Sce I. This might be due to the effect of the gene locus where SCneo was integrated. The reduced number of neo-positive clones was partially restored to normal levels by transient expression of full-length human NBS1 before I-Sce I expression in two clones, nos 17 and 44 (Table 3), indicating that DSB repair through a HR event must be at least partially dependent on the Nbs1 protein. Evidence for the existence of an Nbs1-independent, but DSB-stimulated, recombination pathway is provided by the fact that neo-positive clones were induced only

Table 1 Spontaneous and ionizing-radiation-induced chromosomal aberrations

Genotype	Chromatid		Chromosome		Chromatid exchange	Total aberrations (per cell $\pm$ s.e.)
	Gaps	Breaks	Gaps	Breaks		
Spontaneous						
Wild-type	1	0.5	0.5	0.5	0	0.02 $\pm$ 0.01
<i>Nbs1</i> ^{-/-}	9	4	2	4	1	0.20 $\pm$ 0.04
γ -irradiation induced						
0.3 Gy						
Wild-type						
0–1 h	18	2	2	0	0	0.22 $\pm$ 0.05
2–3 h	6	0	4	0	0	0.10 $\pm$ 0.03
7–8 h	2	0	2	0	0	0.04 $\pm$ 0.02
<i>Nbs1</i> ^{-/-}						
0–1 h	16	12	0	4	0	0.32 $\pm$ 0.06
2–3 h	18	4	4	0	0	0.26 $\pm$ 0.05
7–8 h	18	8	2	2	0	0.30 $\pm$ 0.05
2.0 Gy						
Wild-type						
0–1 h	56	60	2	5	0	1.23 $\pm$ 0.11
2–3 h	13	4	3	3	0	0.23 $\pm$ 0.05
7–8 h	7	2	5	0	0	0.14 $\pm$ 0.04
11–12 h	2	0	3	0	0	0.05 $\pm$ 0.02
<i>Nbs1</i> ^{-/-}						
0–1 h	78	78	12	6	0	1.74 $\pm$ 0.26
2–3 h	46	56	8	2	0	1.12 $\pm$ 0.21
7–8 h	18	16	2	3	3	0.42 $\pm$ 0.07
11–12 h	9	3	4	2	2	0.20 $\pm$ 0.05

The number of aberrations per 100 cells is presented. At least 100 mitotic cells were analysed in each case. The number of total aberrations per cell $\pm$ s.e. is calculated as $x/N \pm \sqrt{x/N}$ as described previously^{2,3}. Each time duration corresponds the cell population irradiated at cell cycle stages: 0–1, G2/M; 2–3, late S; 7–8, G1 for wild type and G1-early S for *Nbs1*^{-/-}; 11–12, early G1 for wild type and middle G1 for *Nbs1*^{-/-}. Note that a strong G2 block was observed in *Nbs1*^{-/-} cells (slight in wild type) at 6–12 h after 2 Gy irradiation.

Table 3 Recombination frequencies in SCneo reporter construct

Genotype	Plasmid	Total no. of transfected cells	Total no. of G418-resistant	Gene conversion frequency
Wild type no. 6	pBluescript	1.54×10^7	17	$(1.16 \pm 0.29) \times 10^{-6}$
Wild type no. T1	3 × nls I-SceI	2.06×10^7	26,418	$(1.29 \pm 0.29) \times 10^{-3}$
	pBluescript	1.60×10^7	110	$(6.88 \pm 0.80) \times 10^{-6}$
	3 × nls I-SceI	1.50×10^7	30,530	$(2.03 \pm 0.70) \times 10^{-3}$
<i>Nbs1</i> ^{-/-} no. 13	pBluescript	3.2×10^6	0	$<3.1 \times 10^{-7}$
	3 × nls I-SceI	3.2×10^6	23	7.2×10^{-6}
<i>Nbs1</i> ^{-/-} no. 17	pBluescript	6.90×10^6	0	$<1.5 \times 10^{-7}$
	3 × nls I-SceI	9.90×10^6	47	$(4.67 \pm 1.07) \times 10^{-6}$
<i>Nbs1</i> ^{-/-} no. 44	pBluescript	6.00×10^6	0	$<1.7 \times 10^{-7}$
	3 × nls I-SceI	1.24×10^7	0	$<8.0 \times 10^{-8}$
<i>Nbs1</i> ^{-/-} no. 17 + human NBS1*	pBluescript	6.20×10^6	8	$(1.39 \pm 0.61) \times 10^{-6}$
	3 × nls I-SceI	3.50×10^6	169	$(4.79 \pm 0.96) \times 10^{-5}$
<i>Nbs1</i> ^{-/-} no. 44 + human NBS1*	pBluescript	5.70×10^6	7	$(1.13 \pm 0.74) \times 10^{-6}$
	3 × nls I-SceI	5.70×10^6	48	$(8.20 \pm 1.80) \times 10^{-6}$

Gene conversion frequency represents mean $\pm$ s.e. of more than two independent experiments except *Nbs1*^{-/-} no. 13 clone. nls, nuclear localization signal.

*The human NBS1 was transiently expressed by transfecting a human NBS1 expression vector 24–48 h before the transfection of the I-Sce I expression vector.

after expression of I-Sce I in the SCneo-*Nbs1*^{-/-} nos 13 and 17 clones (Table 3). To confirm this, we examined neo-positive clones with Southern hybridization. The SCneo structure in the neo-positive clones generated in the absence of a functional Nbs1 was quite different from that seen in cells competent for normal (wild type) HR. A small fragment (800 bp) of the neo sequence was observed in 66% (14/21) of the *Nbs1*^{-/-} clone no. 17, and 33% (5/15) of the *Nbs1*^{-/-} clone no. 13 (Fig. 3b and Supplementary Information). Because this fragment was not observed in wild-type cells after I-Sce I expression, this particular process may occur at a very low efficiency (at a maximum frequency of 10^{-3} of the normal HR frequencies), or it may be induced by the absence of a functional Nbs1 protein. This is in agreement with the fact that mitomycin-C-induced sister chromatid exchanges occur even in *Nbs1*^{-/-} cells, although they were seen at almost background levels in wild-type cells (Fig. 2c, and data not shown). All the fragments had both 5' and 3'-end marker sequences (chicken ovalbumin marker sequences) which were added to the SCneo construct in order to identify each end of the construct, although the linkage is unknown, and had an Nco I or an I-Sce I site (Fig. 3a).

The generation of the fragment was accompanied by an increase of ploidy. The number of major chromosomes (chromosomes 1–5 and Z, the total number is 12 in wild type) was 16.0 ± 5.6 for neo-positive clones with the fragment, whereas it was 12.4 ± 1.0 for clones without the fragment.

This process could be DSB-dependent, because none of the spontaneously G418-resistant clones in wild-type cells had the small (800-bp) fragment. Most of the 800-bp fragments were accompanied by the 3.8 kilobase (3.8 kb) band indicating an LTGC/SCE (long tract gene conversion or sister chromatid exchange) product (17 in a total of 19 clones), although 2 were accompanied by an STGC (short tract gene conversion) product. We confirmed that these are *bona fide* recombinants, because all the neo-positive products have converted the I-Sce I site to an Nco I site (data not shown). A possible way to generate these fragments is via inter-chromatid crossing over just upstream or downstream of a DSB at the I-Sce I site. Although the fragment could not exist in diploid G418-resistant cells, the increase of ploidy might permit the existence of both the fragment and the neo-positive product. Because Rad51 foci formation, which catalyses the strand invasion

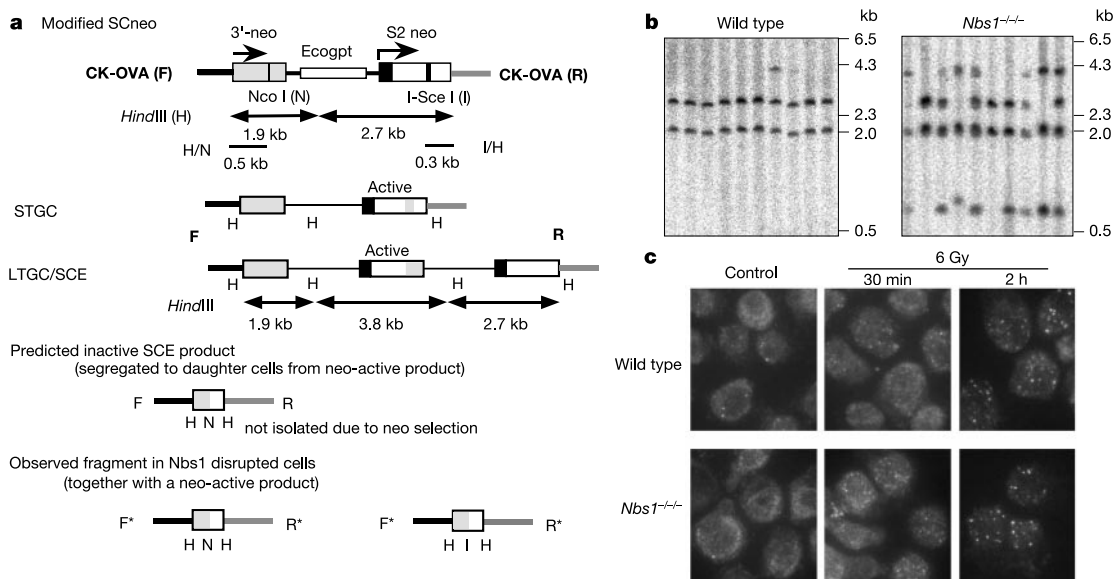


Figure 3 Recombination of the SCneo reporter construct in *Nbs1*^{-/-} cells.

a, Structures of the SCneo. Predicted recombination products (STGC: short tract gene conversion, LTGC: long tract gene conversion²⁴) or observed unusual fragments in *Nbs1*^{-/-} cells are shown. F and R indicate the chicken ovalbumin fragment where

SCneo was inserted. Linkage of F* and R* sides of the unusual fragment is unknown.

b, Southern blot analysis of neo-positive clones. A 800-bp fragment was observed only in *Nbs1*^{-/-} cells. **c**, Rad51 foci formation induced by γ -radiation (6 Gy) was not affected by *Nbs1* disruption. Localization at 0.5 h and 2 h after irradiation is shown.

of HR events, was not affected by Nbs1 disruption (Fig. 3c), the appearance of a recombinant product in SCneo clones with *Nbs1*^{-/-} background raises the possibility that processing or formation of a Holliday junction²⁵ may be affected by Nbs1 disruption, although further experimentation will be necessary to verify this. From these observations, we conclude that the main pathway of HR repair requires a functional Nbs1 complex. To our knowledge, this is the first report that shows the involvement of Nbs1 in DSB repair by HR in vertebrate cells. The Nbs1-dependent HR events could be a reason for a cellular preference for non-crossover events in sister chromatid gene conversion²⁶. □

Methods

Plasmid constructs

The chicken Nbs1 gene (GenBank no. AF230342) was cloned as described previously²⁷. To construct chicken Nbs1 disruption plasmids, 250 bp of genomic DNA including exon 4 was replaced with hygromycin B, puromycin, or blasticidin S selection marker cassettes²⁸ to make the Nbs1-hyg, Nbs1-puro, or Nbs1-bsr constructs. The three Nbs1 alleles in DT40 cells were disrupted using three selection markers.

Cell culture and irradiation

A chicken DT40 cell line was cultured in D-MEM/F-12 medium (GIBCO) supplemented with 5% fetal calf serum (HyClone) and 1% chicken serum (Sigma) at 37°C. DNA transfection and selection was performed as previously described¹⁷. Exponentially growing cells were irradiated with γ -rays using a ⁶⁰Co apparatus (1.0 Gy min⁻¹, Shimadzu) at room temperature. Immediately after irradiation, appropriate numbers of cells were plated in soft agar (0.12%) containing medium. After 7 d incubation at 39°C, the surviving fractions were calculated by comparing the number of colonies formed in the irradiated cell cultures with those in the non-irradiated control.

Western blot analysis and immunostaining

Chicken Nbs1 antiserum was raised in a rabbit against a fusion protein comprising amino acids 607–753 of chicken Nbs1 fused to a 6 × -histidine tag. The antiserum was further affinity purified and used as anti-chicken Nbs1 antibody. Whole-cell extracts (from 2 × 10⁶ cells) were prepared as described²⁷ and 40 μ g of total protein was loaded onto an 8% SDS-polyacrylamide gel. After electrophoresis, immunoblots were performed as described previously²⁷ using anti-chicken Nbs1 antibody. Immunofluorescent staining with anti-Rad51 antibody, provided by A. Shinohara, was performed as described previously²⁷.

DNA synthesis assays

Nbs1^{-/-} cells plated at 2.0 × 10⁵ cells ml⁻¹ in complete medium were irradiated with γ -rays. After 30 min incubation at 39°C, cells were labelled with 1 mCi ml⁻¹ of [³H]thymidine for 30 min. Cells were collected by centrifugation, lysed with 0.4 M NaOH containing 0.2 mg ml⁻¹ salmon sperm DNA, and neutralized with 2 N HCl/sodium pyrophosphate (6%). Each sample was spotted onto a glass filter (Whatman), was washed with 4% trichloroacetic acid and ethanol, and dried using an infrared lamp. Filters were placed into scintillation vials for counting. Averages and standard deviations of the c.p.m. values were determined from triplicate samples.

Chromosome aberration and sister chromatid exchange analysis

Chromosome aberrations and sister chromatid exchange (SCE) analyses were done as previously described³. To analyse mytomycin C (MMC)-induced SCEs, cells were incubated in medium containing 0.05 μ g ml⁻¹ MMC for 12 h. Colcemid, 0.1 μ g ml⁻¹, was added for the last 1.5 h of this incubation before harvesting the cells. Major chromosomes (chromosomes 1–5 and Z) were observed with a microscope for detection of their aberrations or SCE. Karyotype analysis was performed by using photographs of the fixed cells containing distinct chromosomes.

DNA end-joining assay

The plasmid joining substrate pDVH14 was created by ligating oligonucleotides DH5 (5'-GACCATCTACAGCGCTC-3') and DH6 (5'-TCGAGAGCGCTGTAGGATGGTGC-3') between the *HincII* and *XhoI* sites of pDVG42 (ref 29), followed by insertion of oligonucleotides DH7 (5'-CGCGTGATATCCTACAGCTGG-3') and DH8 (5'-GATCCAGCTGTAGGATATCA-3') between the *MluI* and *BamHI* sites. After cleavage with the restriction enzymes *Eco47III* and *EcoRV*, the 7.7-kb vector has a 10-bp direct repeat (ATCCTACAGC) at both ends. The cells were transfected with 2 μ g linear DNA substrate per 2 × 10⁶ cells by electroporation, and incubated for 72 h. The relative contribution of direct end-joining and 10-bp microhomology end-joining (Fig. 2d) was determined by PCR amplification of the junction from extra chromosomal DNA as described³⁰, followed by *XcmI* digestion, giving rise to a labelled 180-bp uncut product and a 120-bp fragment if a *XcmI* site (CCAN₅TGG) has been created at the junction. One of the PCR primers was radiolabelled for easy detection of PCR products. Restriction fragments were separated on a 6% polyacrylamide gel in TBE buffer. The gel was dried, and products were visualized by phosphor imaging. The amounts of the undigested and *XcmI*-digested fragments were quantified using Image Quant software.

Gene conversion and recombination analysis

Gene conversion analysis was performed as described elsewhere^{2,3}. The SCneo construct, provided by M. Jasin, was used for site-specific DNA repair analysis. For this study, the hygromycin-resistant marker for stable transformant selection was substituted with *Ecogpt* (mycophenolic acid resistance), and a fragment containing the genomic sequence of the chicken ovalbumin gene was added to both the 5'- and 3'-ends of the SCneo. The plasmid was transfected into cells by electroporation, and stable transformants with one copy of the SCneo were isolated. For induction of DSBs at the I-Sce I site, pCMV-3xns I-Sce-I²⁴ was introduced into the cells by electroporation. The cells were then incubated for 48 h and subsequently grown in medium containing 1.6 mg ml⁻¹ of G418 (Calbiochem). To distinguish STGC and LTGC/SCE²⁴, genomic DNA was digested with *HindIII* and probed with a fragment of neo DNA.

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Structure of a protein determined by solid-state magic-angle-spinning NMR spectroscopy

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The determination of a representative set of protein structures is a chief aim in structural genomics. Solid-state NMR may have a crucial role in structural investigations of those proteins that do not easily form crystals or are not accessible to solution NMR, such as amyloid systems¹ or membrane proteins^{2–4}. Here we present a protein structure determined by solid-state magic-angle-spinning (MAS) NMR. Almost complete ¹³C and ¹⁵N resonance assignments for a micro-crystalline preparation of the α -spectrin Src-homology 3 (SH3) domain⁵ formed the basis for the extraction of a set of distance restraints. These restraints were derived from proton-driven spin diffusion (PDSF) spectra of biosynthetically site-directed, labelled samples obtained from bacteria grown using [1,3-¹³C]glycerol or [2-¹³C]glycerol as carbon sources. This allowed the observation of long-range distance correlations up to ~ 7 Å. The calculated global fold of the α -spectrin SH3 domain is based on 286 inter-residue ¹³C–¹³C and six ¹⁵N–¹⁵N restraints, all self-consistently obtained by solid-state MAS NMR. This MAS NMR procedure should be widely applicable to small membrane proteins that can be expressed in bacteria.

Solid-state NMR studies are feasible if suitably labelled samples of high structural homogeneity are available. Isotope labelling is a requirement for the applicability of NMR techniques to achieve sequence-specific resonance assignments^{5–7} or to estimate distance restraints and torsion angles^{8–10}. Membrane proteins can be investigated by solid-state NMR using static oriented samples, as was demonstrated with the structure of gramicidin¹¹. Alternatively, MAS applied to oriented or non-oriented samples produces sufficiently resolved spectra and yields isotropic chemical shifts which give rise to amino-acid-specific correlation patterns that are important for assignment strategies, heightening expectations that structural studies of small membrane proteins or their bound ligands—such as 4–60-residue receptor agonists or antagonists—are in principle viable by solid-state NMR^{12–15}. However, a suitable methodology for the collection of a large number of distance restraints in the range of 2–7 Å from a small number of samples and with minimal experimental effort is still lacking. By examining all potential short atomic distances (≤ 7 Å) in proteins and considering the limited resolution of the proton spectrum, it is evident that carbon–carbon restraints are of prime importance for defining a three-dimensional structure. The measurement of such restraints

is hindered in fully ¹³C-enriched samples by dipolar truncation effects^{16,17}. Suppression of these effects can be achieved by applying spectroscopic techniques that contain frequency-selective dipolar recoupling schemes^{9,18,19} or by combining broadband recoupling methods with dilution of ¹³C spins⁸.

We prepared a set of differently labelled samples of the α -spectrin SH3 domain²⁰, which enabled the semi-quantitative interpretation of cross-peak intensities. The set consisted of a uniformly ¹³C-labelled preparation (referred to as U-SH3) and two biosynthetically site-directed ¹³C-enriched samples, obtained by growing bacteria on [1,3-¹³C]glycerol (1,3-SH3) and [2-¹³C]glycerol (2-SH3)²¹. The amino-acid labelling pattern of 1,3-SH3 is shown in Fig. 1a. Amino acids synthesized from precursors made in the glycolysis pathway (group I) show a labelling pattern composed of $\sim 100\%$ (green) and $\sim 0\%$ (red) ¹³C-enriched sites. The inverse labelling scheme is obtained with 2-SH3. A more complex labelling scheme is obtained for the amino acids synthesized from precursors related to the citric acid cycle (group II). Several isotopomers with different labelling patterns are then produced, which are shown in Fig. 1a for the case of proline. The percentage of labelling in each site (Fig. 1a), resulting from the mixture of isotopomers, was estimated from solution NMR data.

The alternating labelling pattern in 1,3-SH3 and 2-SH3 leads to a reduction of dipolar truncation effects and allows the observation of long-range interactions, while relayed polarization transfer is blocked. These effects are demonstrated in Fig. 1b–d, which

Table 1 ¹³C–¹³C and ¹⁵N–¹⁵N distance data and statistics for α -spectrin SH3

(a) Average inter-strand and sequential ¹³ C– ¹³ C and ¹⁵ N– ¹⁵ N distances*			
Inter-strand	Distance (Å)	Sequential	Distance (Å)
Cα _i –Cα _j †	4.6 ± 0.3	Cα _i –Cα _{i–1}	3.8 ± 0.1
Cα _{i–1} –Cα _{j+1}	5.4 ± 0.3	Cα _i –Cβ _{i–1}	4.6 ± 0.3
		Cβ _i –Cβ _{i–1}	5.8 ± 0.4
CO _{i–1} –CO _j	4.9 ± 0.3	CO _j –CO _{i–1}	3.5 ± 0.3
CO _{i–1} –CO _{j+1}	5.0 ± 0.4		
N _{i+1} –N _j	5.1 ± 0.2	N _i –N _{i–1}	3.5 ± 0.2
N _i –N _j	5.8 ± 0.3		
N _{i–1} –N _{j+1}	4.5 ± 0.2		
(b) Restraints and structural statistics for the α-spectrin SH3 ensemble			
Restraints			
Total experimental restraints	292		
Total inter-residue C–C restraints	286		
Sequential ($ i - j = 1$)	122		
Medium range ($1 < i - j \leq 4$)	15		
Long range ($ i - j > 4$)	149		
Restraints in the class 2.5–4.5 Å	10		
Restraints in the class 2.5–5.5 Å	21		
Restraints in the class 2.5–6.5 Å	42		
Restraints in the class 2.5–7.5 Å	213		
Total inter-residue N–N restraints	6		
Restraints in the class $3.0 < r < 6.0$ Å	6		
Distance constraint violations > 0.3 Å	0		
Dihedral angle violations > 20°	0		
Energies			
Final energies (kcal mol ^{–1})	(15)‡		
E _{global}	53 ± 6		
E _{bonds}	1.7 ± 0.4		
E _{angles}	31 ± 1		
E _{impropers} #	1.0 ± 0.2		
E _{van der Waals}	16 ± 4		
E _{dipolar couplings}	3 ± 1		
r.m.s. deviation§			
Deviation from ideal values	(15)		
Bonds (Å)	0.0013 ± 0.0001		
Angles (deg)	0.329 ± 0.008		
Impropers (deg)	0.11 ± 0.01		
Backbone of β-sheet (Å)	1.6 ± 0.3		
(15) versus X-ray for β-sheet elements (Å)¶	2.6 ± 0.2		

*Distances shown are for anti-parallel β -sheets.

†The indices i, j refer to the top left panel of Fig. 2.

‡(15) represents the average for the 15 energy-minimized conformers.

§Evaluated by CNS²⁵.

||The β -sheet comprises residues 7–11, 14–17, 23–26, 29–34, 41–46, 49–54 and 58–61.

¶r.m.s. deviation of the mean structure from the X-ray structure²⁶.

#Impropers are angles for maintaining geometries²⁵.

shows contour plots of two-dimensional ^{13}C - ^{13}C PDSD²² spectra of U-SH3 (Fig. 1b) and of 2-SH3 and 1,3-SH3 (Fig. 1c, d, respectively). All spectra were recorded with a long mixing time of 500 ms to establish long-range correlations. An advantage of the PDSD technique²² is that longer mixing times can be applied without limitations on balancing radio-frequency-power input and sample heating. In Fig. 1b, mainly intra-residue cross-peaks are observed⁵ with additional correlations, attributed to relayed polarization transfers within an amino acid or to transfers between sequentially connected amino acids. The assignment of the correlations in Fig. 1b is, however, difficult owing to overlap and ambiguity. In contrast, the 2-SH3 and 1,3-SH3 spectra (Fig. 1c, d) are simplified owing to the less extensive labelling. The spectrum of the 2-SH3 preparation (Fig. 1c) is virtually empty in the methyl region between 16 and 28 p.p.m. On the other hand, additional peaks are observed particularly in the region from 48–66 p.p.m., which can be assigned to long-range polarization transfer events. The spectrum of 1,3-SH3 (Fig. 1d) shows a large number of new peaks in the methyl region, which can also be attributed to long-range interactions. Positive

side-effects of this type of labelling are the simplification of the assignment of the new correlations and the overall improved resolution as a result of suppressing homonuclear one-bond J -couplings⁸.

We assigned the cross-peaks by applying the criterion that each peak must be part of a correlation pattern involving several nuclei of two interacting amino acids. For example, the assignment of long-range cross-peaks involving L33 and V44 is shown in Fig. 2 (panels I–IV), which contains the regions indicated in Fig. 1c, d. Furthermore, we could identify the secondary structure and topology of the SH3 domain from correlation patterns involving residues 14 and 26, 15 and 25, 16 and 24, or between 34 and 43, 33 and 44, 31 and 46, which indicate the presence of an antiparallel β -sheet. Additionally, at around 62 p.p.m. (Fig. 2, panel V), where the $\text{C}\alpha$ of T24 and P54 resonate, all cross-peaks involving the two sequential α -carbons are observed, as is a correlation between the α -carbon signals of P54 and W41. The inter-strand $\text{C}\alpha$ - $\text{C}\alpha$ correlation between T24 and Q16 is also observed in the same region but only in the 1,3-SH3 spectrum, owing to a low percentage of Q16 $\text{C}\alpha$ -labelling in the

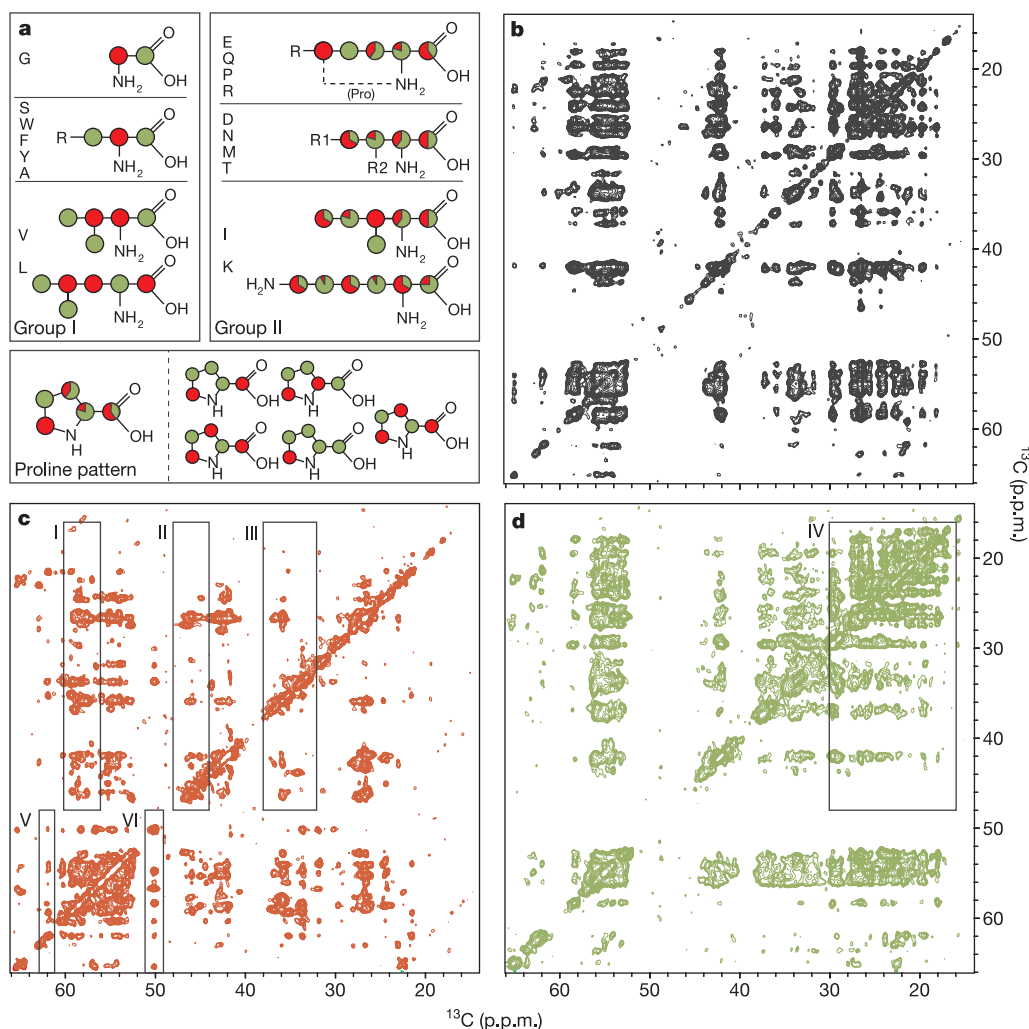


Figure 1 Labelling patterns and NMR spectra for the different α -spectrin SH3 domain preparations. **a**, Schematic representation of the effective ^{13}C enrichment for the indicated residues, as obtained by protein expression in *E. coli* BL21 (DE3). The green colour corresponds to the degree of ^{13}C labelling pattern obtained by growth on $[1,3\text{-}^{13}\text{C}]$ glycerol; the opposite labelling pattern, obtained by growth on $[2\text{-}^{13}\text{C}]$ glycerol, is represented in red. In cases with mixed labelling, the percentage label from $[2\text{-}^{13}\text{C}]$ glycerol and $[1,3\text{-}^{13}\text{C}]$ glycerol for a particular atom is represented using relative red/green colouring. The percentages of labelling for each site were estimated by solution

NMR from satellite signals. The fractional labelling for residues of group II is explained by the production of different labelling patterns, as illustrated for proline at the bottom.

b–d, Contour plots of two-dimensional ^{13}C - ^{13}C PDSD spectra recorded with a mixing time of 500 ms at 17.6 T and a spinning frequency of $\omega_{\text{R}}/2\pi = 8.0$ kHz on U-SH3 (**b**), on 2-SH3 (**c**) and on 1,3-SH3 (**d**). The colours of the spectra (red for 2-SH3 and green for the 1,3-SH3) correspond to the labelling pattern in **a**. The boxed regions (I–VI) in **c** and **d** relate to Fig. 2.

2-SH3 preparation. These observations reflect systematically appearing distances between α -carbons in adjacent strands that range from 4.6–5.4 Å (Fig. 2 top left and Table 1a). Similarly, inter-strand distances between nitrogens, CO and C α are of that order (Table 1a). These characteristic β -sheet cross-peak patterns prove the reliability of the assignment. Likewise, characteristic signal patterns (for example, between residues i and $i + 3$ or $i + 4$) are expected in spectra of α -helical proteins.

To extract distance restraints from the cross-peak intensities, we recorded and analysed a set of spectra with mixing times of 50, 100, 200 and 500 ms. The build-up of the integrated signal intensities was evaluated for a number of reference peaks. Conformation-independent distances between sequential α -carbons, methyl groups within leucine and valine, and α - and γ -carbons within one amino acid and between C β and C γ of the leucines were used as references. In general, the intensities of peaks arising from shorter distances of 2.5 Å built up relatively fast, reaching a maximum at around 100 ms, and then decayed slowly. The build-up curves corresponding to transfers between sequential α -carbons (~ 3.8 Å) increased in the first 500 ms. The time dependence of the signal intensities involving interactions in the 4–6 Å range, such as inter-strand C α –C α interactions in β -sheets, showed lag phases of varying length similar to effects in NOESY spectra of proteins in solution²³. As peak intensities are influenced by several effects, a rigorous quantitative evaluation was not feasible. We therefore categorized the carbon–carbon distance restraints empirically in four restraint classes (Table 1b). We determined the upper distance boundaries of these classes according to the first appearance of the reference peaks at the various mixing times. The reference distance for the first class was defined by cross-peaks between sequential C α atoms (~ 3.8 Å) that appeared within 50 ms, whereas interactions between sequential C α and C β (~ 4.6 Å) and inter-strand C α –C α (4.6–5.4 Å) appeared first

at 100 ms, defining the second class. The third class contained sequential C β –C β interactions (~ 5.8 Å). We assigned all other interactions to the fourth class, with distances in the range of 2.5–7.5 Å. The lower bound was always kept at 2.5 Å, to account for an apparent lower signal intensity due to incomplete suppression of dipolar truncation effects and/or fractional labelling. The carbon–carbon restraints were supplemented by six nitrogen–nitrogen restraints, primarily defining a β -sheet architecture. They were obtained from a single ^{15}N – ^{15}N PDSD spectrum with a mixing time of 4.0 s, and classified in the long-distance range 3–6 Å. Few long-range ^{15}N – ^{15}N correlations were detected because the couplings between ^{15}N spins are approximately sixfold weaker than couplings between carbon spins at comparable distances, and nitrogen atoms form a less dense network relative to carbon atoms.

In solid samples of proteins, intermolecular interactions are expected to cause additional cross-peaks in the PDSD spectra. Initial structure calculations on our SH3 data, for example, showed the necessity of discriminating between inter- and intramolecular contacts on an experimental basis. As the SH3 domain is a small protein, the surface-to-core ratio is too unfavourable to achieve this by analysing the restraint violations. For this reason and as a part of the general concept, we prepared samples containing 80% unlabelled material and 20% 1,3-SH3 or 2-SH3. A set of PDSD spectra with 500 ms mixing time was recorded on these diluted samples in which correlations attributable to intermolecular contacts were reduced approximately fivefold relative to the intramolecular contacts. In this way, contacts between residues Y57–S19, Y57–P20, R21–L8 and P20–Y13 were identified as intermolecular and removed from the restraint list, in line with earlier observations²⁴.

A list of 292 inter-residue distance restraints for residues 7–61 was generated and subjected to a conventional structure calculation

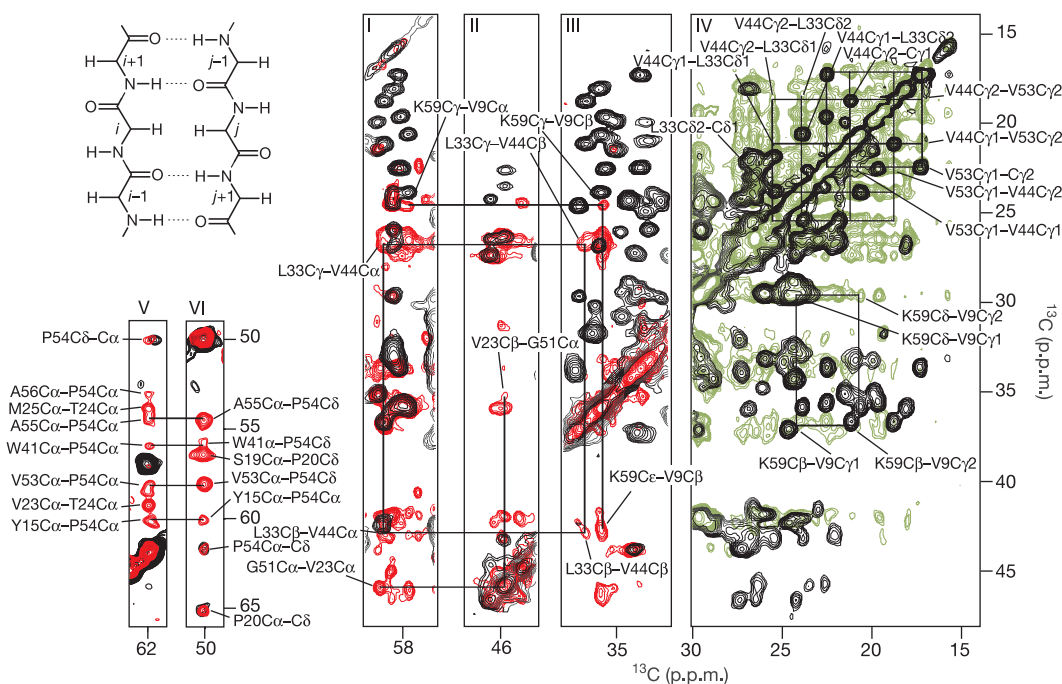


Figure 2 Assignment strategy. Regions extracted from the spectra of Fig. 1c and d (I–VI) superimposed on a PDSD spectrum of uniformly labelled SH3 domain (black), the latter recorded with a short mixing time of 15 ms (ref. 5). Part of the assignment of the long-range correlations is reported in the figure, and the lines define the different correlation patterns. As an example, correlations between residues L33 and V44 are shown. The correlations between the C α and C β signals of V44 and C β and C γ signals of L33 are observed in panels I–III for 2-SH3, whereas correlations between the methyl groups

appear in the spectrum of 1,3-SH3 (panel IV). Of particular interest is the region around 50 p.p.m. (panel VI), where for 2-SH3 a large number of cross-peaks due to the proline- δ signals (P20 and P54) are observed, whereas in the corresponding area of the U-SH3 sample (see Fig. 1b), no correlations are detected. In the upper left corner, a schematic representation of an antiparallel β -sheet is shown. The numbering of the residues (i and j) corresponds to the numbering in Table 1.

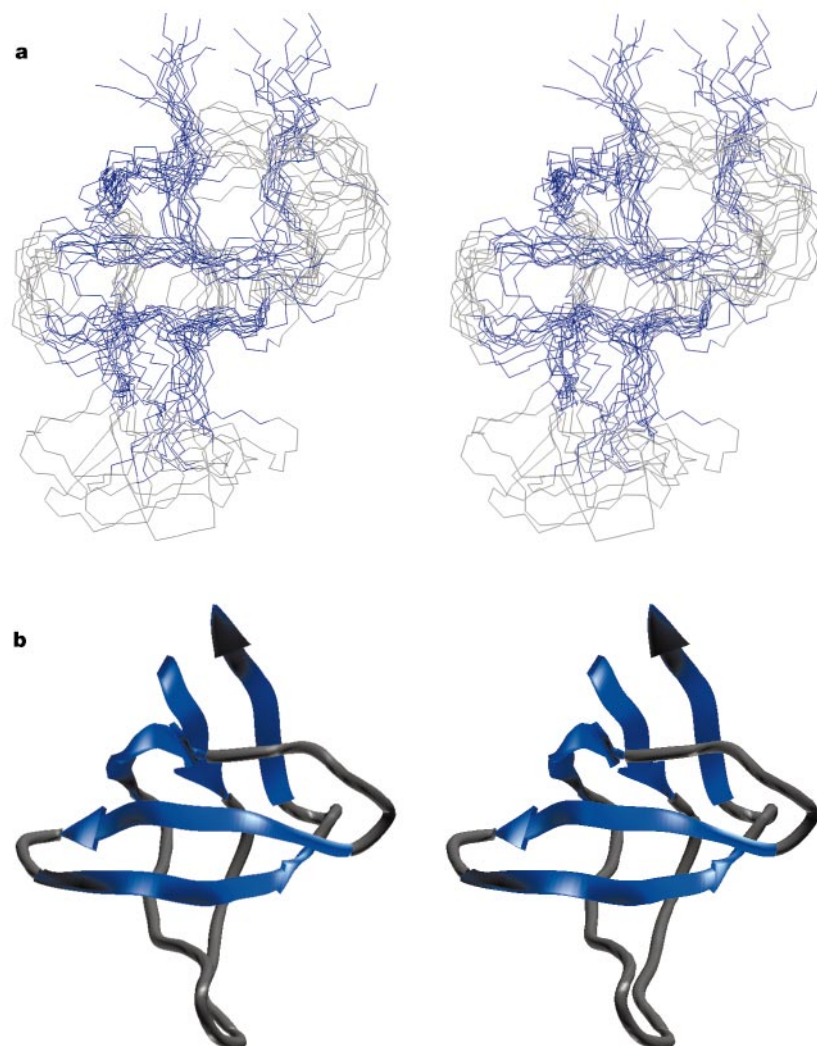


Figure 3 Solid-state structure of the α -spectrin SH3 domain. **a**, Stereo view of twelve of the fifteen lowest-energy structures, representing the fold of the SH3 domain. The three structures with the largest r.m.s. deviation to the average structure are not displayed. The

β -strand regions are shown in blue. **b**, The X-ray structure²⁶ is shown for comparison. In this case, the part of the β -sheet in the region 14–17 and 23–26 is non-ideal and therefore is not indicated in blue.

protocol²⁵. The 15 lowest-energy structures were selected out of 200, which represent the fold of the SH3 domain (Fig. 3a). The X-ray structure is shown below (Fig. 3b) for comparison²⁶. The C α coordinates of the regular structure elements show a root mean square (r.m.s.) deviation of 1.6 Å to the average structure, and of 2.6 Å to the X-ray structure (Table 1b). The coordinates of the grey shaded loop region at the bottom consisting of residues 18–22 show a high degree of divergence in this set of structures; however, we do not attribute this to flexibility but rather to the lack of long-range intramolecular restraints.

We present a solid-state MAS NMR structure that satisfactorily describes the β -sandwich fold of the 62-residue SH3 domain. This result paves the way for the structure determination of amyloids, small membrane proteins or receptor–ligand complexes, provided that the proteins can be expressed in bacteria and suitably labelled⁴. Although the current work is performed on micro-crystalline material, its periodicity is not a requirement for the applicability of the presented methodology, but aids in providing a structurally homogeneous environment. For membrane proteins, structural or conformational homogeneity is supported by either embedding the protein into the membrane or embedding an agonist (or antagonist) into the receptor-binding site, which serves as a matrix and

forces the ligand into a unique environment ensuring sufficiently narrow lines. Our method provides a sufficiently large number of long-range carbon–carbon distance restraints with reasonable precision from dipolar carbon–carbon correlation spectroscopy. The data set may be supplemented by ^1H – ^1H restraints obtained from proton–proton correlation spectroscopy^{10,27} using samples deuterated at non-exchangeable sites, and by ^{13}C – ^{15}N restraints from C–N dipolar correlation spectroscopy using doubly-labelled samples. In the foreseeable future, techniques will be developed to include ^1H – ^{13}C and/or ^1H – ^{15}N restraints. In general, the application of three-dimensional techniques to resolve, for example, carbon–carbon correlation spectra by means of ^{15}N chemical shifts would be necessary to study larger proteins. However, for the small SH3 domain presented here, two-dimensional spectroscopy was sufficient. The study of bacterial membrane proteins within structural genomics projects is an obvious next application of the new technique described herein, together with the study of receptor-bound agonists and antagonists. □

Methods

Sample preparation

Plasmid pET3d coding for α -spectrin SH3 protein from chicken brain was a gift of

M. Saraste. The SH3 protein was expressed in *Escherichia coli* BL21 (DE3), using M9 minimal medium. A total of 0.5 g $^{15}\text{NH}_4\text{Cl}$, 2.0 g $\text{NaH}^{12}\text{CO}_3$ and 2.0 g $[1,3\text{-}^{13}\text{C}]\text{glycerol}$ per litre of medium was added in the case of $[1,3\text{-}^{13}\text{C}]\text{glycerol}$, ^{15}N SH3. For the preparation of the $[2\text{-}^{13}\text{C}]\text{glycerol}$, ^{15}N SH3 sample, 0.5 g $^{15}\text{NH}_4\text{Cl}$, 2.0 g $\text{NaH}^{13}\text{CO}_3$ and 2.0 g $[2\text{-}^{13}\text{C}]\text{glycerol}$ were used²¹. The proteins were purified as previously described²⁰. The final yield was approximately 20 mg of protein per litre of culture. A 200 mM $(\text{NH}_4)_2\text{SO}_4$ solution (pH 3.5, 0.04% NaN_3) was added to a 1.15 mM SH3 solution (pH 3.5) at a volume ratio of 1:1. The protein samples for MAS measurement were precipitated as previously described²⁰.

NMR spectroscopy

All solid-state carbon–carbon NMR experiments were performed at a field of 17.6 T on a DMX-750 narrow-bore spectrometer, equipped with a 4-mm double-resonance MAS probe (Bruker, Karlsruhe). The protein was confined to the centre of the rotor with the use of spacers. Approximately 6–7 mg of protein was used in all experiments, except for the fivefold diluted samples, where 9–10 mg of protein was used. All two-dimensional correlation spectra were acquired at MAS frequencies of 8.0 kHz or 13.0 kHz using time-proportional phase incrementation for phase-sensitive detection. Ramped cross-polarization from ^1H to ^{13}C created the initial transverse carbon magnetization; spin-lock fields were 36 kHz for ^1H and 18–36 kHz for the ^{13}C ramp. After the first ^{13}C evolution period, carbon magnetization was exchanged by using a PDS mixing scheme²². Spin diffusion periods of 50–500 ms were applied. Typical carbon 90° pulse lengths were 5.3 μs . A proton RF field of ~ 60 kHz was applied for the two-pulse phase modulation decoupling²⁸ during ^{13}C acquisition and evolution. The two-dimensional ^{13}C – ^{13}C spectra were recorded with 32–64 scans, and with ~ 6 ms evolution in the indirect dimension, leading to experimental times of 16–32 h. The spectra of the 80% unlabelled and 20% labelled protein were recorded with 128 scans in 2.5 days. The ^{15}N – ^{15}N PDS spectrum was recorded under similar experimental conditions, but with a mixing time of 4 s and on a DMX-750 wide-bore spectrometer, equipped with a 4-mm triple-resonance MAS probe (Bruker, Karlsruhe).

The data were processed with the XWINNMR software version 2.6 (Bruker, Karlsruhe) and subsequently analysed using the program Sparky version 3.100 (T. D. Goddard & D. G. Kneller, University of California).

Structure calculations

Structures were calculated with the program CNS version 1.0 (ref. 25). Calculations were performed using the simulating annealing protocol with torsion-angle dynamics, starting with 200 randomized conformers. The $286\text{ }^{13}\text{C}$ – ^{13}C restraints were categorized into strong (2.5–4.5 Å), medium (2.5–5.5 Å), weak (2.5–6.5 Å) or very weak (2.5–7.5 Å) classes. The six ^{15}N – ^{15}N correlations were constrained to 3–6 Å. Twelve of the fifteen lowest-energy structures with no violations larger than 0.3 Å were used to represent the three-dimensional fold of the α -spectrin SH3 domain. The atomic coordinates and NMR restraints were deposited in the RCSB Protein DataBank under entry number 1M8M.

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Absolute comparison of simulated and experimental protein-folding dynamics

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Protein folding is difficult to simulate with classical molecular dynamics. Secondary structure motifs such as α -helices and β -hairpins can form in 0.1–10 μs (ref. 1), whereas small proteins have been shown to fold completely in tens of microseconds². The longest folding simulation to date is a single 1- μs simulation of the villin headpiece³; however, such single runs may miss many features of the folding process as it is a heterogeneous reaction involving an ensemble of transition states^{4,5}. Here, we have used a distributed computing implementation to produce tens of thousands of 5–20-ns trajectories (700 μs) to simulate mutants of the designed mini-protein BBA5. The fast relaxation dynamics these predict were compared with the results of laser temperature-jump experiments. Our computational predictions are in excellent agreement with the experimentally determined mean folding times and equilibrium constants. The rapid folding of BBA5 is due to the swift formation of secondary structure. The convergence of experimentally and computationally accessible timescales will allow the comparison of absolute quantities characterizing *in vitro* and *in silico* (computed) protein folding⁶.

To establish a statistically favoured mechanism, map the free energy surface, and to compare absolute rate and equilibrium constants with experimental data requires multiple simulations of any folding reaction. Such multiple sampling has only been achieved for peptide systems^{7–10}. As an alternative, simulations in the past have been compared to relative quantities such as Φ -values¹¹, or high temperature unfolding rates². The prevailing view is that current molecular dynamics cannot find the native state at the free energy minimum owing to limitations of timescale and force field accuracy. For example, performing a molecular dynamics simulation for 10 μ s with a 2-fs time step on a simple system in implicit solvent, such as our model protein BBA5, would require decades for a typical modern CPU. Our approach is to compare directly experimental rate and equilibrium constants with distributed computing simulations of the same quantities from thousands of short (5–20 ns) folding trajectories totalling hundreds of microseconds. Absolute quantities such as rate constants provide strong constraints on barriers and stringent folding model tests, but are more difficult to calculate precisely than relative quantities. For instance, agreement between simulation and experiment within a factor of two would be noteworthy for even small-molecule rate prediction. To allow experiment and theory to meet on the microsecond timescale, we have chosen to study mutants of the fast-folding, designed mini-protein BBA5. The choice of a 23-residue protein with strong secondary structure propensities and a small hydrophobic core reduces the impact of force field inaccuracies, and allows kinetics to be resolved both experimentally and computationally.

Although folding during a 20-ns simulation is improbable, collecting a very large number of trajectories has allowed us to observe such improbable events (Fig. 1). Given a small, two-state protein that obeys exponential kinetics with a mean folding time of 10 μ s, roughly 10 out of 10,000 individual molecules should have folded conformations after 10 ns (ref. 10). Individual trajectories may not be arbitrarily short. Each simulation must first relax from the initial condition to the unfolded state under native conditions. For BBA5 this relaxation is extremely rapid—the initial extended ensemble collapses to a compact unfolded ensemble within 10 ns. The unfolded ensemble under native conditions has a significant degree of α -helical structure (in both simulation and experiment). Our simulations also satisfy the constraint that each simulation should not be substantially shorter than the minimal passage time through the transition state (about 10 ns, from peptide diffusion data^{12,13}). The Folding@Home distributed computing project¹⁴, a cluster of over 30,000 volunteer computers distributed around the world, has been used for several months to accumulate approximately a million CPU days of simulation time (<http://folding.stanford.edu/>). We observed folding in over 100 independent trajectories. Calculations and laser temperature-jump experiments indicate that our BBA5 mutants are moderately cooperative folders, have a very low melting temperature, and fold on a timescale of several microseconds. Furthermore, the simulations characterize the timescale for the formation of secondary structure, and demonstrate folding by means of a heterogeneous transition state (Supplementary Information).

Two mutants of BBA5 (Fig. 1a), a 23-residue β -hairpin/turn/ α -helix motif inspired by the zinc finger fold, were constructed on the basis of the sequence developed by the Imperiali group, Ac-YRVpSYDFSRDELAKLLRQHAG-NH₂ (ref. 15). BBA5 is a 'mini-protein' containing all three elementary units of secondary structure, and folds without obligate metal-ion binding¹⁶. A D-proline residue favours the formation of a type II' β -turn, stabilizing a hairpin centred about residues 4–5. The amino-terminal β -hairpin and carboxy-terminal α -helix interact to form a small hydrophobic core. Native state simulations at 278 K reveal flexibility in the relative orientation of the hairpin and the helix. Our single mutant replaces a phenylalanine at position 8 with a tryptophan, which acts

as a fluorescent probe and as a larger aromatic residue for interaction with side chains in the helix. The double mutant additionally replaces valine 3 with a tyrosine. Both mutants show the same basic fold in simulations, and have proton NMR spectra with dispersion similar to BBA5. BBA5 mutants were chemically synthesized, purified and buffered in 10 mM phosphate at pH 7 for all experiments. To study the folded mutants, homology models were constructed from the structure of BBA5 (see Methods).

Circular dichroism and fluorescence spectra of BBA5 mutants were measured to quantify the folding equilibrium (Fig. 2). The folding transition was weakly cooperative, as expected for a mini-protein (midpoint below 0 °C). To assess secondary structure changes during folding, far-ultraviolet circular dichroism spectra were collected from –15 °C to 90 °C. Both mutants fold with increased helical content at low temperature (local minimum at 222 nm). Their circular dichroism spectra closely resemble BBA5 (ref. 15). The N-terminal 1–10 hairpin fragment by itself has a random coil spectrum without cooperative transition (Fig. 2a). The C-terminal 11–23 helix fragment has higher secondary structure propensity. To assess solvent exposure of the core, tryptophan fluorescence as a function of wavelength was measured at temperatures from –2.5 °C to 60 °C. On unfolding at higher temperature, the fluorescence intensity of the double mutant displayed a redshift of 11 nm, indicating increased tryptophan solvent exposure. The fluorescence and circular dichroism spectra have identical melting transitions, as well as isosbestic/isodichroic points where all spectra intersect. This indicates that the BBA5 mutants fold thermodynamically by means of a two-state mechanism. The calculated equili-

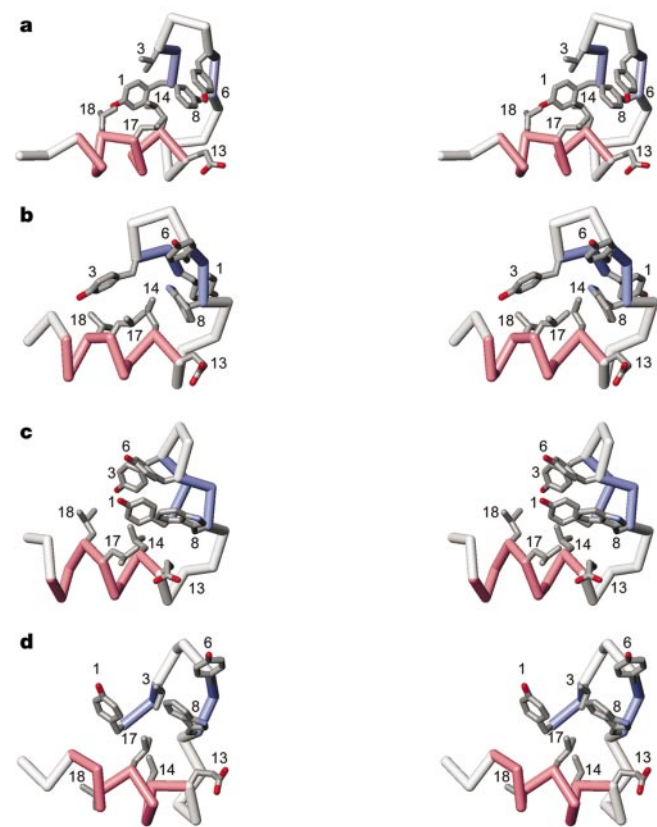


Figure 1 Folding results. Stereo diagrams showing the C α backbone (blue, 1–3 and 6–8; red, 11–21) and selected side chains (Tyr 1, Val/Tyr 3, Tyr 6, Phe/Trp 8, Glu 13, Leu 14, Leu 17 and Leu 18) for folding trajectories terminating with low r.m.s.d._{C α} to BBA5. **a**, NMR structure of BBA5. **b**, Double mutant at 2.2 Å. **c**, Double mutant at 2.4 Å. **d**, Single mutant at 2.5 Å. See Supplementary Information for examples of folding trajectories.

brium constant K_{eq} for the double mutant is shown as a function of temperature in Fig. 2c (see also Methods).

To investigate experimental folding kinetics, laser temperature jumps of $11 \pm 1^\circ\text{C}$ were performed on both BBA5 mutants with final temperatures between 293 and 303 K. Relaxation to the new equilibrium concentration was monitored at 100-ns intervals by following the temporal shift of the fluorescence spectrum with a sub-microsecond, real-time multichannel fluorescence wavelength detector¹⁷. The double mutant response could be resolved above the 400-ns instrument response function (Fig. 2d; see also Methods).

The same $1.5 \pm 0.7 \mu\text{s}$ relaxation rate was detected whether analysing intensity changes or wavelength shifts of the time-resolved fluorescence spectrum. Standard definitions for the folding rate and

the equilibrium constant of a two-state folding protein resulted in a folding rate of $7.5 \pm 3.5 \mu\text{s}$ at 298 K (see Methods). For the single mutant we can establish a limit $\tau < 10 \mu\text{s}$. The kinetic data are entirely compatible with single exponential folding kinetics, although we cannot exclude processes of small amplitude, or speed greater than the 400-ns resolution of our apparatus. Such processes could become relevant for fast folding over a low barrier (for example, a subpopulation of protein molecules might fold directly without need for further thermal excitation to the barrier).

Our choice of a tractable protein, a united atom model, an implicit solvent treatment, and the use of distributed computing techniques allowed aggregate simulation of over 700 μs . Folded state simulations were started from homology models (see Methods). We acquired 2,500 single-mutant folded simulations (10 ns) at both 278 K and 298 K. We also acquired 2,500 double-mutant folded simulations (10 ns) at 278 K, 378 K and 478 K. Twenty-five alternative double-mutant models generated 8,750 trajectories (5 ns) at 278 K. Folding trajectories were started from extended initial conformations. A total of 15,000 single-mutant folding simulations (20 ns) were acquired at both 278 K and 298 K; 9,000 and 8,500 double-mutant folding simulations were acquired, respectively, at 278 K (20 ns) and 338 K (10 ns). Considering all 32,500 folding trajectories, we observed the expected β -hairpin in over 1,100 independent trajectories and the α -helix in over 21,000 independent trajectories. We selected successful folding trajectories that possessed both native secondary structure and low alpha carbon position root mean square deviation (r.m.s.d._{C α}) with BBA5 (see Methods). Of 9,000 double-mutant folding trajectories at 278 K, 16 were folded after 20 ns (Fig. 3). Trajectories that fold quickly are not unphysical¹⁰; the fact that the average unfolded molecule explores conformational space for microseconds before finding the native conformation does not preclude individual molecules from folding quickly. Quite the opposite, the basic principles of two-state protein kinetics indicate that we must expect to observe a small fraction of molecules to fold on short timescales¹⁸.

Our simulations were consistent with the experimentally determined folding rate and stability. Our two-state assumption was justified by the thermodynamic data. The interpolated simulated folding time at 298 K was about $6 \mu\text{s}$ for the double mutant, and we computed unfolding rates slightly higher than the folding rates. This is to be compared with the experimental folding time of $7.5 \pm 3.5 \mu\text{s}$ and K_{eq} of 0.25 ± 0.1 (that is, the unfolding rate k_u is about four times faster than the folding rate, k_f). The primary uncertainty in the computed rate was not the fit, but sensitivity to the folding criteria; varying the r.m.s.d._{C α} cutoff by $\pm 0.5 \text{ \AA}$ resulted in expected folding times at 298 K of 3–13 μs . Simulations of the single mutant at 298 K were more difficult to resolve and generated an expected

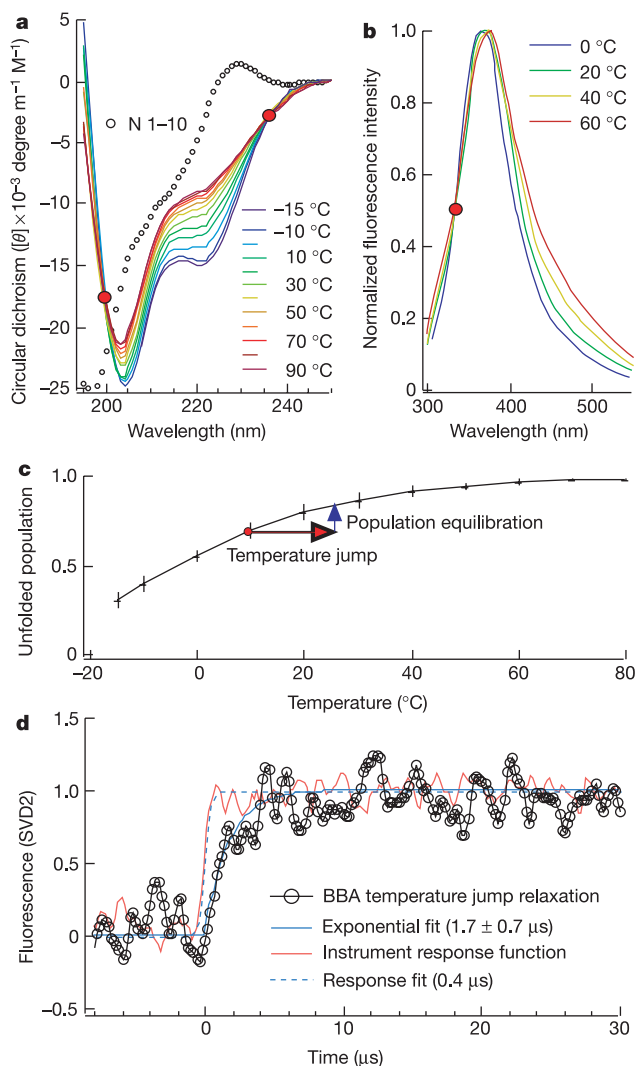


Figure 2 BBA5 double mutant thermodynamics and kinetics. **a**, Circular dichroism spectra from -15°C to 90°C . Isodichroic points (red circles) and the largest singular values ($S2/S1 = 0.10$, $S3/S1 = 0.02$, $S4/S1 = 0.02$, $S5/S1 = 0.01$; see Methods) indicate thermodynamic two-state behaviour. The N-terminal fragment is a random coil. Circular dichroism spectra of the single mutant (not shown) are very similar. **b**, Intensity normalized fluorescence spectra. The emission peak shifts from 366 nm to 377 nm and fluorescence intensity decreases by 2.5 as temperature increases from 0°C to 60°C . The 330-nm isosbestic point is further evidence of a two-state mechanism. **c**, Unfolded protein population with error bars. **d**, Temperature-jump relaxation kinetics and tryptophan instrument response at an average temperature of 298 K. The kinetics are normalized second singular value components of the time-evolving fluorescence spectrum ($\Delta\nu \approx 2 \text{ nm}$). The single exponential fit with a relaxation time constant of $1.7 \mu\text{s}$ yields a deconvoluted relaxation time constant of $1.5 \mu\text{s}$.

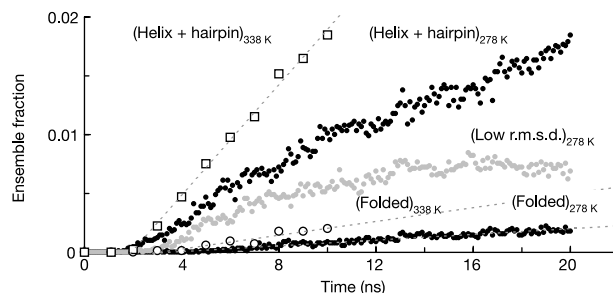


Figure 3 Folded population growth. The growing population of folded double mutant conformations, at 278 K (filled circles, dotted line) and at 338 K (open circles, dotted line), the population with low r.m.s.d._{C α} (grey circles), and the population with both helix and hairpin at 278 K (filled circles) and 338 K (open squares, dotted line), are shown. The scale of the y axis illustrates the need to simulate thousands of trajectories. Note the increased rate of folding at high temperature.

folding time of 16 μ s (7–43 μ s), which was roughly consistent with experimental observation ($\tau < 10 \mu$ s). A total of 10,000 trajectories at a timescale of tens of nanoseconds seem sufficient to provide a reasonable statistical estimate of the folding rate for this rapidly folding, two-state mini-protein.

It is important to address directly limitations in terms of methods and modelling. First, to discriminate folded structures, we rely on qualitatively reasonable but arbitrary criteria. Also, our simulations do not represent structure prediction, as our folded criterion relies on the C α positions of BBA5. Besides methodological limitations, it is imperative to address whether established methods, such as the optimized potentials for liquid simulations (OPLS) parameter set, the generalized Born surface area solvent model, and stochastic dynamics within Tinker are able to sample native-like states without further optimization. The observed native state flexibility may be an indication that our force field was insufficiently accurate to describe completely the native structure free energy minimum. Finally, given a 400-ns instrument response function and a 1.5- μ s relaxation rate, the uncertainty in the experimental folding rate is still large.

There is a growing body of knowledge about rapid protein dynamics. The fastest helices in apomyoglobin form in approximately 50 ns (ref. 19), and a collapsed hydrophobic core in the same molecule forms in about 10 μ s (ref. 20). It has been suggested that helices and turns commonly form on the timescale of about 100 ns and 1 μ s, respectively¹. For the BBA5 single mutant, we can make the rough estimate that α -helix formation above the level in the unfolded state population has a relaxation time of about 500–800 ns (from 278 to 298 K). We further estimate that the β -hairpin has a relaxation time of about 1.7–1.5 μ s (from 278 to 298 K). The β -hairpin forms more quickly in the double mutant with a relaxation time of only about 0.5 μ s at 278 K, which demonstrates the sensitivity of simulated folding kinetics to a single mutation and explains why the estimate of the double-mutant folding rate exceeds the single mutant estimate. These folding rates approach the theoretical maximal folding rate estimated from protein chain diffusion¹². The ability to simulate an approximately 10- μ s process by sampling many shorter trajectories puts any number of rapidly folding proteins within our reach. Simulated rates could complement and explain quantities correlated with rate, such as contact order²¹.

The two principal challenges of simulated protein folding—to build accurate models and to adequately sample conformational space—are not easily decomposed. Here, we have addressed an improved means of sampling; using many thousands of independent trajectories, we have explored the conformational space of two BBA5 mutants. We have observed their folding many times, a process that takes microseconds. We have studied the behaviour of ensembles of thousands of trajectories. Helical structure in the unfolded state, fragment secondary structure propensity, rate of helix formation, rate of hairpin formation, and the rate for folding were all consistent with the available experimental evidence. The ability to investigate directly folding dynamics and heterogeneity with molecular dynamics will bring molecular dynamics to the same prominence in protein folding that it has already achieved in structural modelling. □

Methods

Protein preparation

BBA5 mutants and fragments were synthesized with C-terminal amidation and N-terminal acetylation by Bioworld or the UIUC Biotech Center to 70% purity. High-performance liquid chromatography (HPLC) purification using a Vydac C18 reversed-phase analytical column yielded over 90% purity, verified by mass spectrometry. The samples were lyophilized after purification. To calculate concentrations, extinction coefficients at 280 nm were estimated for the mutants and fragments using the extinction coefficients of tyrosine and tryptophan residues. C-terminal fragment concentrations were determined by amino-acid composition analysis, using the Moore–Stein method²².

Model preparation

The BBA5 double mutant model 1 was generated by homology to BBA1 (Protein Data Bank code 1HCW)¹⁶ with 74% sequence identity. All applicable torsion values from the

most representative member of the BBA1 NMR ensemble were taken for an initial conformation. Mutant residues used the Tinker²³ default torsions of 180° except for Trp 8 where a side-chain torsion angle χ_1 of 0° relieved a steric clash. No memory Broyden–Fletcher–Goldfarb–Shanno (BFGS) quasi-Newton energy optimization²⁴ to r.m.s. gradient convergence, followed by 100 ps of molecular dynamics, provided a numerically stable initial conformation. The other double mutant models (2–26) were generated by homology to BBA5 (91% identical to the double mutant)¹⁵. We took each of the 25 NMR structures, removed Val 3 and Phe 8, placed Tyr 3 and Trp 8 simultaneously into their most favoured rotamers (holding the rest of the protein fixed) using a self-consistent mean field method²⁵, and minimized each structure including a generalized Born surface area energy term²⁶ at each step. To obtain an initial unfolded structure, a fully extended conformer was generated using Tinker (backbone torsions $\phi, \psi = -135, 135$). A total of 100 steps of minimization relieved a clash between the side chain and carbonyl of D-Pro 4. This extended structure was equilibrated for 100 ps using molecular dynamics, providing a numerically stable denatured structure. The single mutant and fragment simulations were started from similarly prepared extended conformations.

Circular dichroism and fluorescence measurements

Fluorescence spectra were excited at 285 nm in a 1-cm quartz cuvette at 100 μ M peptide concentration. A thermocouple and circulating water bath measured temperature to $\pm 0.2^\circ$ C. Sixty micromolar circular dichroism samples (0.3–2 mm cuvettes) were temperature equilibrated for 1 min for each temperature point. In addition to isosbestic points, singular value decomposition (SVD) was used to verify that the thermodynamic data could be modelled as a function of temperature with only two significant species; the third and higher SVD basis functions were negligible (see legend to Fig. 2): two species account for most of the observed circular dichroism and fluorescence spectral changes with temperature. The second SVD component was fitted to $K_{eq}(T)$, with and without baselines, to estimate the population error from inaccurate baselines (Fig. 2c). $K_{eq} = \exp[-\Delta G_f(T)/RT]$, where $\Delta G_f(T) = mT(T - T_m)$, ΔG_f is folding energy, T is temperature, $R = 8.31 \text{ J K}^{-1} \text{ mol}^{-1}$ and T_m is the melting temperature of BBAW ($-2 \pm 3^\circ$ C). The resulting unfolded fraction $(1 - K_{eq})/(1 + K_{eq})$ (Fig. 2c) changes from under 25% to over 75% over a 45 $^\circ$ C range.

Temperature jumps

Temperature jumps of 11 $^\circ$ C were induced by a 1.5- μ m wavelength infrared pulse lasting 10 ns, generated by a Raman-shifted YAG laser. Free tryptophan in phosphate buffer was used to calibrate the temperature jump before each measurement series. Time-resolved, dispersed fluorescence data was taken by channelling the fluorescence (excited by 288-nm pumping with a frequency-tripled, mode-locked Ti:sapphire laser) by means of a liquid ultraviolet light guide through a monochromator, which imaged 60 nm of the spectrum (340–400 nm) into 15 channels of an array photomultiplier tube¹⁷. The net unfolding process was monitored by an approximately 11-nm fluorescence redshift. SVD as a function of time also revealed only two significant basis functions in the time-evolving fluorescence spectra. The measured relaxation rate was $k_o = k_f + k_u$, where $K_{eq} = k_f/k_u$. The reported rate of 1.5 μ s was obtained by deconvoluting the 400-ns gaussian full-width-at-half-maximum instrument response from a 1.7- μ s fit.

Molecular dynamics

Our simulations used software adapted from the Tinker molecular modelling package²³ and the OPLS united atom parameter set²⁷. Constant temperature stochastic dynamics modelled the viscous drag of water ($\eta = 91 \text{ ps}^{-1}$). We modelled solvation with the generalized Born/surface area implicit solvent model²⁶. The electrostatic calculations used 16 Å cutoffs with 12 Å tapers. The bond lengths were constrained with the RATTLE algorithm allowing time steps of 2 fs²⁸. His 21 was given neutral charge. Trajectory conformations were recorded at intervals of 0.1–1 ns.

For analysis, a conformation contained α -helix if it had at least four α -helical residues according to the program Dictionary of Protein Secondary Structure (DSSP)²⁹ with the default H-bond cutoff parameter of 0.5 kcal mol⁻¹. A conformation contained the native hairpin if it had a hairpin with β -bridges between residues 2–7 and 3–6 according to DSSP. To quantify the presence of expected tertiary structure, we aligned each conformation to the C α positions of the reported low energy BBA5 NMR structure and calculated r.m.s.d._{C α} using ProFit. A conformation had low r.m.s.d._{C α} if it had r.m.s.d._{C α} with BBA5 < 3.622 Å (one standard deviation above the equilibrated (10 ns) folded double-mutant ensemble mean). A conformation had high r.m.s.d._{C α} if it had r.m.s.d._{C α} with BBA5 > 5.0 Å (one standard deviation below the final unfolded ensemble mean). A conformation was folded if it had the helix, the hairpin, and low r.m.s.d._{C α} . A conformation was unfolded if it lacked either the helix or the hairpin, and had high r.m.s.d._{C α} .

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Supplementary Information accompanies the paper on *Nature's* website (<http://www.nature.com/nature>).

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Competing interests statement The authors declare that they have no competing financial interests.

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erratum

Simulation of the atmospheric thermal circulation of a martian volcano using a mesoscale numerical model

Scot C. R. Raffkin, Magdalena R. V. Sta. Maria & Timothy I. Michaels

Nature **419**, 697–699 (2002).

In this Letter, “(see Supplementary Information)” should have appeared at the end of the third sentence of the third paragraph. At the end of the Letter, the line “Supplementary Information accompanies the paper on *Nature's* website (<http://www.nature.com/nature>).” should have been included. □

corrigendum

Undermethylation associated with retroelement activation and chromosome remodelling in an interspecific mammalian hybrid

Rachel J. Waugh O'Neill, Michael J. O'Neill & Jennifer A. Marshall Graves

Nature **393**, 68–72 (1998).

In this Letter, the maternal species listed for hybrid BE-1 is attributed to *Macropus eugenii*. However, our ongoing studies show that the maternal complement of chromosomes in BE-1 was inherited from a *Macropus rufogriseus* female. The centromeres of *M. rufogriseus* chromosomes are extended in comparison to all other macropod species. The extent, therefore, to which the centromere extensions shown in Fig. 4 can be attributed to hybrid-specific amplification of the retroelement KERV-1 cannot be precisely determined. Nevertheless, Southern analysis confirms that this retroelement is present at a 20% higher copy number in the hybrid's genome compared with that of its parents, and FISH analysis shows KERV-1 localization only to centromeres in the hybrid. Our conclusions regarding hybrid-specific undermethylation in this hybrid individual are not affected because *M. rufogriseus* shows methylation levels typical of species within the macropod group. Hybrid-specific undermethylation and genome rearrangement also remain true for the *Petrogale* hybrids we presented. □

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Learning from misconduct

There are some obvious and not-so-obvious career lessons to be learned from the misconduct case of Jan Hendrik Schön. The physicist was fired from Bell Laboratories in Murray Hill, New Jersey, after an investigation determined that many of his publications on superconductivity in carbon 'buckyballs' contained fabricated data.

The obvious lesson is that misconduct does not pay. Before he was caught, Schön had achieved fame for publishing a flurry of high-profile papers that eventually proved too good to be true. But the revelations of fraud have ruined a once-promising career. The process by which he was found out shows how science — albeit sometimes slowly and clumsily — inevitably ferrets out such fraud. Suspicions arose when several scientists failed to duplicate his results, prompting a closer look at his data, and ultimately revealing that plots and graphs from different experiments were identical, even down to the noise.

The less obvious lesson is that preventing and policing misconduct is the responsibility of everyone in the scientific community. The immediate aftermath of the Schön affair resulted in the predictable blame game. Now that the dust has started to settle, the community can take the next step to prevent future incidents.

During a conference on research integrity at the Institute of Medicine in Washington last month, Steven Teitelbaum, president of the Federation of American Societies for Experimental Biology, said that mentors should be the first to take that step. Teaching research integrity should be an integral part of a mentoring relationship. And monitoring their charges' data is even more important. Because although misconduct can ruin the perpetrator's career, it can also taint the reputation of their mentor.

Paul Smaglik
Naturejobs editor



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MOVERS

Research gets a fresh focus at Keck School of Medicine; Paradigm genetics welcomes new president; pharmaceutical director comes out of retirement; and more [Back page](#)

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SCIENTIFIC EVENTS

Transitions

X Greg Fahiman, executive director of the Canada-France-Hawaii Telescope, will leave that position in January to become director general of the Herzberg Institute of Astrophysics in Victoria, Canada.

X Donna Dean has been named the first deputy director of the National Institute of Biomedical Imaging and Bioengineering in Bethesda, Maryland, which opened this year.

X John Mitchell will replace **Paul Mason** who is retiring next month as chief scientist of the UK Met Office in Bracknell, Berkshire. Mitchell, currently head of modelling climate change at the Hadley Centre, has worked for the Met Office for 29 years.

X Tom Tuschli is moving to Rockefeller University from the Max Planck Institute for Biophysical Chemistry, Göttingen, Germany.

X This month Brian Dynlacht left Harvard University to head the genomics programme at New York University.

X Last month Richard Armer, former section head of medicinal chemistry at Glasgow-based Organon Laboratories, joined Ardara Bioscience in Edinburgh as head of chemistry.

X Jean-Jacques Bienaimé has been appointed chief executive and president of Palo Alto-based Genencor International. He succeeds **Thomas Mitchell**, who will continue as chairman of the board.

X Wayne Gombotz has been named vice-president, process sciences and pharmaceutical development, at Corixa, a Seattle-based biotech firm. Previously he was a senior director at Immunex, another Seattle-based company.

MEDICINE

A first degree in English, even from Yale University, isn't the main qualification that springs to mind for a top post at a medical school. But neuroscientist **Zach Hall** — the new senior associate dean for research at Keck School of Medicine at the University of Southern California — has never been afraid to try something new.

It wasn't long before Hall added a PhD from Harvard University in biochemistry to his initial English degree. Since then he has worked in academia, industry and government (including a period as director of the National Institute of Neurological Disorders and Stroke), written a textbook and been a founding member of the journal *Neuron*. His latest brainchild, EnVivo, was created in what he calls "a very interesting year" to launch a biotech company.

Finding that each sector operates in different ways with different objectives, he says that "recognizing and developing talented people is key in all of these institutions".

Hall has big plans at Keck, aiming to expand the research base by recruiting 135 faculty members over the next eight years.

DRUG DISCOVERY

Retirement lasted just four months for **Claes Wilhelmsson**. The former executive director and head of R&D at AstraZeneca took up a new post last month as chairman of Gyros, an Uppsala-based company that is developing microfluidics technology to create laboratory tests on compact discs.

Wilhelmsson began his career in 1969 at the Sahlgrenska Hospital in Gothenburg, then joined Astra in 1985 as medical director for cardiovascular diseases.

He was tempted to work at Gyros by its technology, which could speed up drug discovery — as well as by the opportunity to stay active in science and business while enjoying the benefits of semi-retirement. There's ample opportunity for other pharmaceutical managers willing to make a similar switch, he says. "There's a huge pool of start-up biotech companies in Europe. Some of them need big pharma experience for their board membership."

INTEGRATIVE GENOMICS

Gene-mapping pioneer **David Botstein** will leave Stanford University for Princeton in New Jersey next July, to replace **Shirley Tilghman** as director of the Lewis-Sigler Institute for Integrative Genomics. Botstein led efforts to map and sequence the yeast genome, advised the Human Genome Project and pioneered the use of microarrays to analyse cancer-gene expression. He will move into new lab



Claes Wilhelmsson



David Botstein



Heinrich Gugger



Robert Waterston

space housing 12–15 research groups specializing in post-genomic science.

Botstein taught at the Massachusetts Institute of Technology (MIT) for 20 years, then served as vice-president of San Francisco biotech company Genentech before joining Stanford.

"The emergence of the data from the Human Genome Project completely changes the way biology can and will be done," he says. "The question of what kind of preparation young people should have in order to enter into this exciting new world requires serious thought."

He is moving to follow his interest in combining teaching and research. At MIT he developed a series of undergraduate courses he called "project labs", which focus on research questions that can be answered with the most up-to-date techniques. He plans to create similar courses at Princeton.

AGROCHEMICALS

Heinrich Gugger, the new president and chief executive of Paradigm Genetics in Research Triangle Park, North Carolina, knows a thing or two about restructuring. He led a merger between Novartis Crop Protection and Zeneca Agrochemicals to form Syngenta Crop Protection. He joined Paradigm when it was cutting staff from 280 to 200. "Now we are managing a turnaround," Gugger says.

Gugger, who started his career at the University of Bern in Switzerland, joined Paradigm to become more involved as a bridge between science and business. After the merger that formed Syngenta, the balance tilted towards sales and marketing. "It took away some of the stuff I was fond of," says Gugger.

GENOME SCIENCES

Robert Waterston, who played a key role in mapping and sequencing the human genome, is to leave Washington University in St Louis, Missouri, for the University of Washington in Seattle in January. He will chair the university's department of genome sciences. On top of his contributions to the Human Genome Project, Waterston has set widely followed new standards for managing research centres in emerging fields.

Waterston led the sequencing of the first animal genome, the nematode *Caenorhabditis elegans*. His laboratory constructed the genetic map that the Human Genome Project used as a data scaffold, and also contributed about 20% of the project's sequence data. Waterston has been a proponent of making sequence data readily available over the Internet.

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